THE MEASUREMENT OF THE EFFICIENCY OF DIETS. NEW APPARATUS AND PROCEDURES [1]

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FOUR FIGURES

(Received for publication November 8, 1937)

Within the memories of the middle-aged of today nutrition was understood to be a comparatively simple process, and nutritive values of foods were measured, with general confidence and satisfaction, in terms of calories, digestible nutrients, metabolizable energy or net energy.

During the current century, however, the science of nutrition has been, in effect, born anew—so great has been the advance in our knowledge; and, as each revolutionary discovery has followed the one before, thus contributing to our understanding of the subject as one of vast complexity, the idea of evaluating foods in terms of common measures has been pushed so far into the background as to appear to be ever less and less practicable and significant.

In reality, however, human society has not outgrown the need for measures of food value; we deal with foods in a quantitative way; foods are obtainable in only limited amounts; and nutritive values in relation to cost will remain always a consideration of first-class economic importance.

An especially baffling feature of this whole matter has been the growing appreciation, to which this laboratory has contributed a long series of studies, of the fact that foods cannot be scientifically evaluated, individually, in any common terms, since each essential nutrient, at least finally, if not immediately, affects the utilization of every other.

[1] Authorized for publication on November 4, 1937, as paper no. 793 in the journal series of the Pennsylvania Agricultural Experiment Station.

It seems to be a fact, however, that nutritive values can be measured, with some degree of consistency as of the more or less completely balanced diets in which foods are used; and it is therefore important that there be generally accepted, standard methods of evaluation of diets, especially as means of expression—in common terms—of the effects of specific nutritive deficiencies.

In this light the present study was undertaken, in a spirit of seeking practical, conventional methods of dietary comparison and evaluation, based, as definitely as practicable, upon scientific understanding and method.

The immediate objective in this investigation was the demonstration of the applicability of the carbon and nitrogen balance method to small animal experimentation, and the comparison of two plans of study of dietary values by this method, with the albino rat as the experimental subject.

EXPERIMENTAL DIETS

Six diets, of significance with reference to human nutrition, were compounded in such manner that each would have approximately the same nitrogen and gross energy values; and these diets were compared, as above stated, by two radically different plans of calorimetric experimentation, depending, in both cases, on the carbon and nitrogen balance procedure, which, in the course of many years' work with cattle, at this institute, has yielded essentially the same values for heat production as has direct calorimetry.

The diets were compounded from the following constituents:

Diet no.	*Components*
1	Wheat and whole milk powder
2	Bread, butter, beef and roots
3	Bread, butter, beef, and green vegetables
4	Bread, butter, beef and fruits
5	Peanuts, fruits and green vegetables
6	Corn meal, navy beans, pork cracklings and molasses

The percentage composition of the diets is given in table 1. Diet no. 1 was exceedingly simple, but, normally, of nearly maximum nutritive value; nos. 2, 3 and 4 consisted of meat,

TABLE 1

Composition of diets[1]*: air-dry basis*[2]

Experiment no. 1	Diet no.	Wheat	Milk powder	NaCl	Protein	Cal. per gm.							
	1	45.16	53.85	0.99	18.25	4683							
		Peanut	Celery	Green beans	Lettuce	Spinach	Peach	Prune	Orange juice	Tomato	NaCl	Protein	Cal. per gm.
	5	30.15	5.45	5.45	5.45	5.45	9.41	9.41	14.12	14.12	0.99	18.44	4634
Experiment no. 2		Wheat	Milk powder	NaCl	Protein	Cal. per gm.							
	1	47.10	51.91	0.99	17.44	4631							
		Beef	Bread	Butter fat	White potato	Sweet potato	Carrot	Beet	NaCl	Protein	Cal. per gm.		
	2	9.03	50.07	8.22	19.01	6.34	3.17	3.17	0.99	17.13	4601		
		Beef	Bread	Butter fat	Celery	Green beans	Lettuce	Spinach	NaCl	Protein	Cal. per gm.		
	3	5.24	72.93	7.17	3.42	3.42	3.42	3.42	0.99	17.06	4612		
		Beef	Bread	Butter fat	Peach	Prune	Orange juice	Tomato	NaCl	Protein	Cal. per gm.		
	4	8.67	59.84	7.23	4.65	4.65	6.98	6.98	0.99	17.56	4620		
		Peanut	Celery	Green beans	Lettuce	Spinach	Peach	Prune	Orange juice	Tomato	NaCl	Protein	Cal. per gm.
	5	30.15	5.45	5.45	5.45	5.45	9.41	9.41	14.12	14.12	0.99	18.44	4634
		Corn meal	Pork cracklings	Navy beans	Molasses	NaCl	Protein	Cal. per gm.					
	6	58.76	22.37	11.35	6.53	0.99	17.69	4695					

[1] All values are per cent except those for calories per gram.

[2] All products were in the dried (air-dry) condition, except for molasses, which contained its normal percentage of moisture.

bread and butter, as basic constituents, each supplemented with a different type of vegetable food; no. 5 was composed of vegetable products alone, with roasted peanuts as the main source of protein; and no. 6, of mixed animal and vegetable origin, possessed a certain superficial acceptability, but was probably highly inadequate in some fundamental respects.

In this outline of the nature of the diets, the 'roots' were white potato, sweet potato, carrot and beet; the 'green vegetables' were celery, string beans, lettuce and spinach; and the 'fruits' were peach, prune, orange juice and totato—all in the dried (air-dry) form.

The beef preparation was made by drying and pulverizing fresh beef, which had been freed from visible fat and connective tissue. After grinding, in a meat chopper, the fresh meat was heated on a steam bath to a temperature sufficient to accomplish slight cooking, in order to prevent spoilage during drying, after which it was dehydrated at about 65°C. in a hot air oven, and was then finely ground.

PLAN OF EXPERIMENTATION

The general background data relating to experiments nos. 1 and 2 are given in tables nos. 2 and 3, respectively.

The method of the first experiment was to compare two of the diets, nos. 1 and 5, by means of two groups of young rats selected as for paired feeding, in growth and metabolism studies, an unusual condition of this experiment being that exactly the same quantity of food (6.2 gm. per day) was given to each rat, from the beginning to the end of the test.

Three-day periods of metabolic measurement were conducted, by the carbon and nitrogen balance method, at intervals of 2 or in some cases 3, or 4 weeks, there being seven such periods with diet no. 1, and four with diet no. 5.

In the second experiment the six diets described in table no. 1 were compared, each with a different group of six, young, growing, albino rats, selected for uniformity of age and weight; and it was planned that each such group would be subjected to the same series of five metabolic measurements,

by the carbon and nitrogen balance procedure, as indicated below:

1. Fast; preliminary feeding, at the maintenance level, during 6 days, followed, after 1 day, by a fasting heat measurement during 24 hours.

TABLE 2

Experiment 1: Schedule of experimentation

RESPIRATION PERIOD NO.	DIET NO. AND CONSTITUTION	AGE OF RATS [1]	WEIGHT OF RATS	FRESH WEIGHT OF FOOD	DRY WEIGHT OF FOOD	NITROGEN OF FOOD	CARBON OF FOOD	ENERGY OF FOOD
		weeks	*gm.*	*gm.*	*gm.*	*mg.*	*gm.*	*Cal.*
1	1. Wheat, milk powder	7	110	6.2	5.747	181	2.770	29.03
2	1. Wheat, milk powder	9	129	6.2	5.747	181	2.770	29.03
3	1. Wheat, milk powder	11	146	6.2	5.747	181	2.770	29.03
4	1. Wheat, milk powder	13	163	6.2	5.747	181	2.770	29.03
5	1. Wheat, milk powder	15	178	6.2	5.747	181	2.770	29.03
6	1. Wheat, milk powder	19	192	6.2	5.747	181	2.770	29.03
7	1. Wheat, milk powder	22	195	6.2	5.747	181	2.770	29.03
1	5. Peanuts, dried fruits, green vegetables	8	96	6.2	5.995	183	2.770	28.73
2	5. Peanuts, dried fruits, green vegetables	10	109	6.2	5.995	183	2.770	28.73
3	5. Peanuts, dried fruits, green vegetables	12	119	6.2	5.995	183	2.770	28.73
4	5. Peanuts, dried fruits, green vegetables	14	129	6.2	5.995	183	2.770	28.73

NOTE: Six rats were used with each treatment in each period, except that with diet no. 2, in periods nos. 3 and 4, only three and two rats, respectively, were used.

[1] At time of respiration measurement.

2. Maintenance; preliminary feeding, at the maintenance level, 4 days; followed by excreta collection during 7 days, and respiration measurement during 3 days.

3. Growth; preliminary feeding, on the production level, 4 days, followed by excreta collection during 7 days, and respiration measurement during 3 days.

4. Maintenance; the time intervals and treatments the same as for the first maintenance period.

5. Fast; heat measurement during 24 hours, beginning 1 day after the termination of the preceding respiration measurement at the maintenance level.

In the conduct of this experiment, however, a departure was made from this schedule, in one important detail, regarding the preliminary feeding for the first period (fast), which will be discussed later.

TABLE 3

Experiment 1: Partition of average energy consumed per rat per day[1]

PERIOD	FEED	FECES	URINE	PROTEIN RETAINED	FAT RETAINED	HEAT PRODUCTION	FECES AND URINE	BODY GAIN	LIVE WEIGHT
				Diet 1: milk powder, wheat					
	Cal.	*Cal.*	*Cal.*	*Cal.*	*Cal.*	*Cal.*	*Cal.*	*Cal.*	*gm.*
1	29.03	2.53	1.23	2.62	2.28	20.37	3.76	4.90	110
2	29.03	2.63	1.34	1.74	3.27	20.06	3.97	5.01	129
3	29.03	2.55	1.48	1.32	2.12	21.57	4.03	3.44	146
4	29.03	2.73	1.42	1.58	1.66	21.64	4.15	3.24	163
5	29.03	2.84	1.45	1.37	0.18	23.19	4.29	1.55	178
6	29.03	2.69	1.56	0.77	— 0.22	24.24	4.25	0.55	192
7	29.03	3.06	1.68	0.38	0.20	23.71	4.74	0.58	195
				Diet 5: peanuts, fruits, green vegetables					
1	28.73	6.79	1.27	1.10	1.42	18.15	8.06	2.52	96
2	28.73	6.52	1.46	0.94	1.24	18.57	7.98	2.18	109
3	28.73	5.80	1.56	1.16	1.37	18.84	7.36	2.53	119
4	28.73	5.78	1.60	1.05	0.12	20.19	7.38	1.17	129

[1] Each value is an average of results obtained with six rats.

THE CARBON AND NITROGEN BALANCE METHOD

The procedure used for the measurement of the heat production, known as the carbon-and-nitrogen balance method, depends on 1) a measured and constant food intake, 2) the collection and analysis (carbon, nitrogen and energy) of the urine and feces, and 3) the measurement of the CO_2 eliminated by the lungs.

From the gain of nitrogen are computed the equivalent quantities of protein, carbon and energy gained.

From the intake of carbon are subtracted the quantities of carbon eliminated in urine, feces and CO_2, and the carbon content of the protein gained, the remainder being the carbon gained as fat.

Finally, the heat production is computed by subtracting from the energy intake the energy of the feces and urine, and of the energy gain as protein and as fat.

An illustration of this computation follows:

Daily balance

	DRY MATTER	NITROGEN	CARBON	ENERGY
	mg.	*mg.*	*mg.*	*Cal.*
Feed		181	2770	29.03
Feces		26	290	3.25
Urine		146	143	1.64
CO_2			2293	
Protein	54	9	28	0.31
Fat	21		16	0.20
Heat production				23.63

The special advantage of the carbon and nitrogen balance method is that it permits the measurement of the total effect of a diet on the metabolism of an animal, including values for protein and fat gained or lost, as well as the heat production, for protracted periods, under normal conditions as to physical activity.

A basic assumption involved—that the carbohydrate content of the body is the same at the end as at the beginning of the balance period—has been found during more than a quarter century of experimental work at this institute, with cattle, in similar, normal states of nutrition, to be fully warranted; and even though there were a change in the carbohydrate content of the body, the resultant errors in the computed heat production and body gain of energy would be slight, since the energy values of the carbon of fat and carbohydrate are closely similar.

DESCRIPTION OF APPARATUS AND ROUTINE OF OPERATION

The apparatus used for determining the carbon and nitrogen balances, shown in figure 1, consisted of a cylindrical, water-sealed chamber, made of tin-coated, sheet copper, inside which the rat was confined in a cage constructed of ⅓ inch mesh galvanized iron screen.

This cage was 8 inches in diameter, and 10 inches high, with a false bottom of ½ inch mesh galvanized iron screen placed 1 inch above the lower edge of the cage. During use this cage was set into a crystallizing dish, the inside dimensions of which were 8⅜ inches diameter by 2¾ inches height.

The lower edge of the cage was held 1¼ inches above the bottom of the crystallizing dish by three wires soldered to the cage in such position as to support it from the edge of the dish; and below the cage a ⅛ inch mesh stainless steel wire screen, supported from the bottom of the dish, retained the feces, and permitted the urine to fall through to the bottom, from which it was collected.

The feeding cup and holder were constructed as described and illustrated in an earlier paper by Forbes, Swift, Black and Kahlenberg ('35).

During the determination of the metabolizable energy of a diet, prior to the conduct of a respiration experiment, the rat serving as the subject was confined in this cage by an inverted pie tin placed over the top.

During respiration measurements the rat and cage were placed inside the chamber shown in the figure.

This chamber was provided with two windows, 4 inches × 6 inches in dimensions. The windows were of celluloid, cemented onto brass window frames. These frames were cut from 1/16 inch sheet brass, and were soldered inside the copper cylinder, the opening in the cylinder being ½ inch larger, each way, than the opening in the brass plate forming the window frame, thus leaving a ¼ inch edge of brass plate exposed on each side of the opening. The celluloid window pane was cemented onto this exposed ¼ inch edge of brass plate.

The passage of a ventilating air stream through the chamber was provided for by an entrance tube in the side wall at the height of the false bottom in the inside cage, and an exit tube near the top of the side wall.

Food was introduced into the feeding cup, during a respiration experiment, through a copper funnel, soldered into the top of the cylinder. This funnel was made by 'spinning' in order that it would be smooth inside, and could be tightly closed with a rubber stopper.

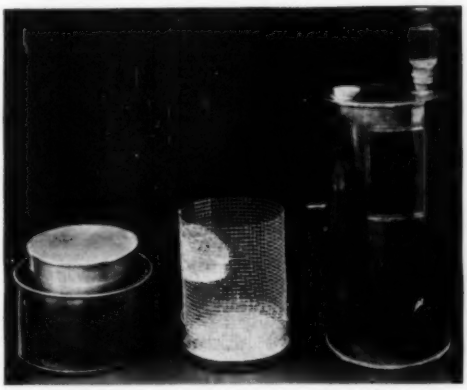

Fig. 1 Respiration chamber, used to determine the carbon and nitrogen balance.

During feeding, the ventilation was stopped, and, before the feeding funnel was opened, the internal pressure was allowed to equalize with the outside pressure, through the soda-lime tube—the internal pressure being slightly less than the outside pressure, by an amount equivalent to a 2-inch column of water.

The animal was watered by the device shown in the illustration. The watering tube, the lower end of which is visible through the window (fig. 1) passes through two rubber stoppers, one of which closes the bottle, and the other is held by a collar of brass tubing soldered into the top of the cylinder.

During a respiration experiment air was drawn through the chamber at the rate of 0.75 liter per minute. The air flow

was regulated to a rate measured by means of a manometer, containing water, with a capillary tube inlet in the suction line. This device was calibrated with the aid of a graph made by establishing a number of points from which a curve was drawn representing rise of water in the suction arm of the manometer corresponding to rate of air flow through the apparatus.

In the establishment of each point on this curve, representing a different rate of air flow, the rate of flow (abscissa) was first measured with a Bohr meter, attached at the intake end of the absorption train; and then, with the Bohr meter replaced by the regulating device, but with the same suction prevailing, the rise of water in the manometer (ordinate) was measured.

During the determination of metabolizable energy, before the cage was placed inside the respiration chamber, the separate collection of urine and feces was made essentially as described in an earlier paper, by Swift, Kahlenberg, Voris and Forbes ('34).

The urine was preserved, during the determination of metabolizable energy, by $CuSO_4$ and NaF previously introduced into the crystallizing dish in solution, and then evaporated to dryness. Twenty grams NaF and 40 gm. $CuSO_4.5H_2O$ were made up to 2 L with water, and 50 cc. of this solution was used in each collection dish.

The urine was washed out of the dishes with hot water, at the end of the collection period, and, after being made up to a definite volume, aliquot samples were taken for carbon and nitrogen determination.

The energy of the urine was computed from its carbon content, extensive data from this laboratory having shown that with diets containing at least 15% of protein the energy-carbon ratio is essentially constant at 11.5 Cal. per gram carbon.

During the determination of metabolizable energy the feces were removed daily from the stainless steel wire screen. The feces of each rat were kept separate until the end of the collection period, after which they were air dried, and weighed.

The feces of the six rats on a given diet were then combined, in order to make a sample sufficiently large for analysis; but the weight of feces from each individual rat was employed in computing the quantities of carbon, nitrogen and energy eliminated therein.

In the experiments to be discussed six of the respiration chambers were used as shown in figure 2, thus providing for the use of six animals, as repeats, on the same diet.

The accessory equipment requires no description except to list the parts, in the order in which the air stream passes through them: H_2SO_4 bottle, soda-lime tube, respiration

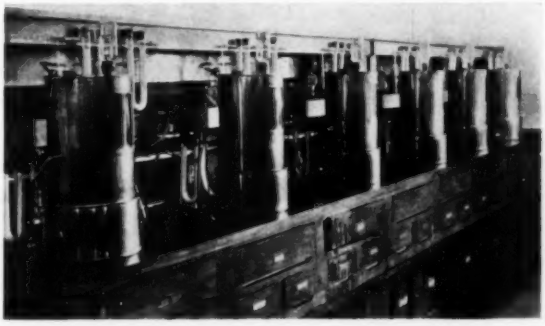

Fig. 2 Respiration chambers equipped as in use.

chamber, H_2SO_4 bottle, acid-pumice check tube, two soda-lime tubes, two acid-pumice tubes, vacuum pump. Also there must be a by-pass for 'sweeping out' the chamber, by providing that the air shall pass directly from the chamber to the vacuum pump.

It was found that during a respiration experiment about 10.4 gm. of water were given off from the water seal into the air stream, but that no CO_2 passed through the water seal.

During feeding, as distinguished from fasting periods, urine and feces were collected only while the cage was outside the respiration chamber, since, in the chamber, the feces remained moist, and underwent offensive decomposition—when handled

in the usual way. During CO_2 measurement, therefore, the stainless steel screen was removed from the crystallizing dish, and the excreta were allowed to drop into a solution of $CuSO_4$ and NaF, covered by mineral oil, in the crystallizing dish.

During fasting periods the respiration chambers were 'swept out,' through by-passes, for 1½ hours before the beginning of the 24-hour CO_2 measurements. The fasting urine, therefore, was collected during 25½ hours; and, after the urines from the six rats on a single treatment had been combined and analyzed, the results were computed to the basis of one rat for 24 hours.

Since the average 24-hour urinary outgo of carbon, nitrogen and energy represented only a total of 6 rat days, however, these values were employed in only a tentative manner in the computation of the heat production.

For comparison with the values thus obtained the heat production was also computed from the CO_2 production, by the use of an assumed respiratory quotient of 0.74, since it had been found, by Kriss, Forbes and Miller ('34), that this R.Q. prevails under such conditions as existed.

The heat production as computed by the second procedure was found in all cases to be lower than as computed by the first method, the average difference being 3.7% of the smaller quantity. The values computed by the second procedure were considered as probably more nearly correct, and these values alone are reported, and utilized in computations.

In the conduct of an experiment, the collection of CO_2 in the first respiration chamber of the series of six was started at 10.00 A.M., and, thereafter, in the remaining chambers at intervals of 15 minutes; and since the first chamber was swept out for 1½ hours, the other chambers were swept out for increasing periods of time reaching 2¾ hours for chamber no. 6.

At the end of each 24-hour period the ventilation of each respiration chamber was stopped, for about 10 minutes, to permit the weighing of the absorption tubes. The CO_2 which accumulated during this time was then accounted for in the course of the next experimental day.

DISCUSSION OF RESULTS

Experiment no. 1

This may be regarded as a growth experiment, with results expressed in the form of the familiar growth curve, supplemented by a series of carbon and nitrogen balances, which revealed the progressive changes in the partition of the food energy, and in the character of the growth.

The method of feeding, however, differed, as already stated, from the ad libitum procedure commonly followed in growth experiments, in that the same amount of feed was given throughout the test.

This procedure afforded a uniquely searching basis for comparison of the values of the diets, since, as the animal increased in size, it was subjected to an increasingly rigorous test of its ability to maintain itself and to grow on a food supply, which, under the circumstances, must have been progressively less and less adequate to the animal's needs.

The results of experiment no. 1 comprise table 3, and are graphically represented in figures 3 and 4, which exhibit a marked contrast between the values of the two diets used, nos. 1 and 5.

With diet no. 1 the intervals between the seven balance periods were 2, 2, 2, 2, 4, and 3 weeks, respectively, while with diet no. 5 the intervals between the four periods were each 2 weeks. The placing of the balance periods, in point of time, is indicated in the graphs by the separate, short lines, apart from the growth curves.

The partition of the energy of the diets shows that the most prominent difference between their nutritive values was in the digestibility of the energy producing nutrients—as indicated by the caloric equivalent of the feces.

The superiority of diet no. 1 to no. 5 was also indicated by the smaller amounts of energy in the urine, the larger amounts retained as fat and as protein, and the larger gains in live weight.

The heat production from diet no. 1 exceeded that from diet no. 5, but the proportion of the metabolizable energy eliminated as heat from diet no. 5 exceeded that from diet no. 1.

At the termination of period 4 the rats on diet no. 5 were killed. The hair was rough, and the general appearance unhealthy. The feces were small, numerous and black. The

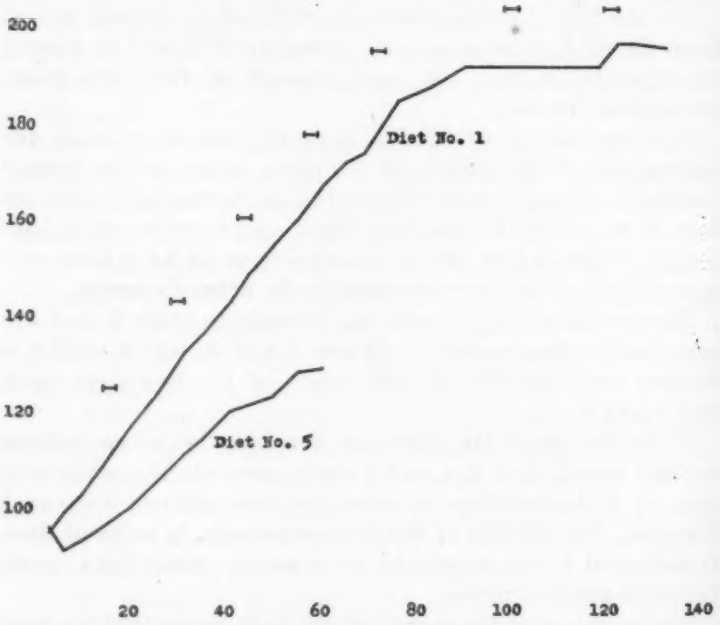

Fig. 3 Curves representing the average growth of six rats, with a constant rate of food intake, on each of two diets.

eyes were light colored; the caeca were distended, and nodules were present on the small intestines.

Of especial interest are the changes which took place in the disposal of the constant food energy as the experiments progressed. Thus, the daily energy gain, in the case of diet no. 1, decreased from 4.90 Cal. per day, in period 1, to 0.58 Cal.

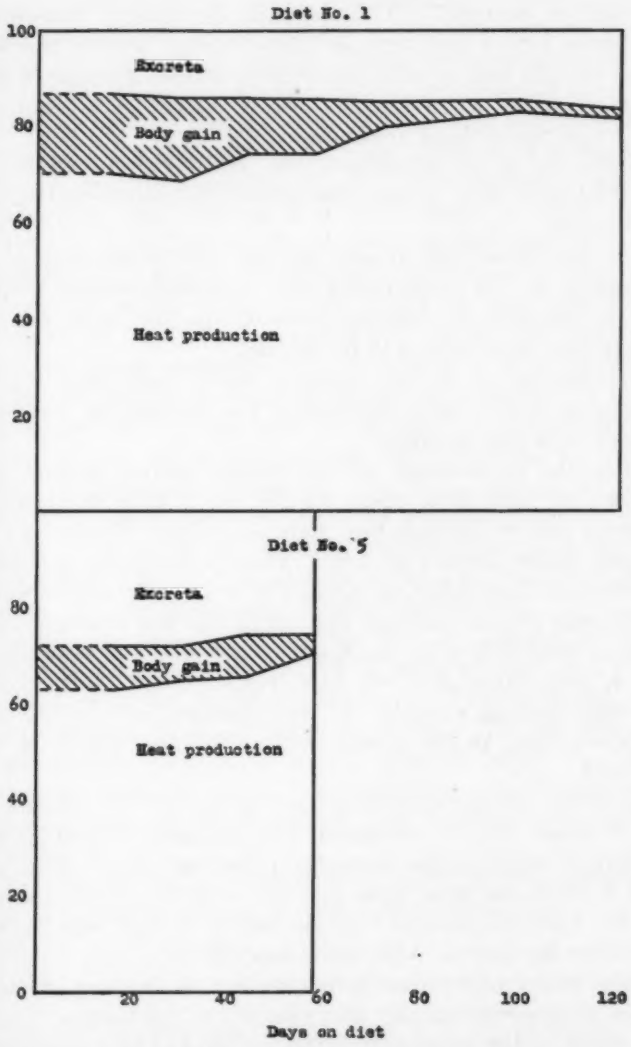

Fig. 4 Representing the average partition of the food energy of six rats, with a constant rate of food intake, on each of two diets.

per day, in period 7. This decrease in gain is necessarily associated with a like increase in the energy leaving the body in the excreta and as heat, about 23% of the decrease in gain being accounted for by an increase in energy of excreta, and 77% by increased heat production.

At the end of the experiment the food intake was sufficient but little more than to keep the animal in protein and energy equilibrium.

It is also important to note that the heat production at the beginning of the experiment was relatively much greater than at the end, in consideration of the fact that the live weight increased from 110 to 195 gm.

The regularity in the changes which took place in the disposal of feed energy throughout the experiment, as shown in table 1, is also notable.

After the termination of the fourth balance period with the rats on both diets, those on diet no. 1 were continued in order to follow through the changes in food disposal as the animals made further growth. Thus, a decrease took place in body gain from 1.55 Cal. to 0.55 Cal. over a period of 4 weeks.

The rats which received diet no. 5 did not consume their feed satisfactorily after the first two periods; and in periods 3 and 4, only three and two rats, respectively, conformed to the experimental routine. It was characteristic of the diet, therefore, that, in the course of time, it came not to be well accepted.

The total energy gained by each group of rats during the first 8 weeks may be computed by assuming a constant rate of change between the bi-weekly points of observation. On such a basis the total gain per rat of those which received diet no. 1 during the first four periods (8 weeks) was 243 Cal. For those on diet no. 5 the gain was 126 Cal.

Total gains might also be determined, at the end of such a series of observations, by analysis of the rat bodies.

A study of the results from 168 rat days of this experiment shows that the CO_2 measurements under the prevailing conditions, which allowed the animal the freedom of the cage, were satisfactorily concordant.

Among fifty-four values for daily CO_2 outgo, computed from 3-day respiration measurements, and three such values for 2 days (no data being excluded), the average deviation was 1.1% of the mean, the greatest deviation being 2.8%.

In a preliminary test period with six rats of 100 gm. average live weight it was found that 5.0 gm. daily of diet no. 1 was sufficient exactly to maintain their live weight during 14 days.

Assuming this maintenance requirement to vary as the 0.73 power of the live weight, we may compute that the 6.2 gm. fed in this experiment would permit growth up to a weight of 134 gm., at which time the rat would be forced to use all the feed for maintenance, without growth. However, the intake of 6.2 gm. actually allowed growth up to a live weight of 195 gm., which evidence of increasing efficiency of utilization of food energy may be considered as at least in part due to diminishing use of food energy for maintenance. Maintenance requirements of energy, therefore, should be determined with the subjects as nearly as possible in energy equilibroum. The profound effect of undernourishment on basal metabolism (per unit of surface area) has long been known (Zuntz, '13; Magnus-Levy, '06; Chittenden, '04).

In this comparison of the two diets of equal protein and energy contents, one composed of milk powder and wheat, and the other of roasted peanuts, dried fruits and dried vegetables, the first diet mentioned sustained the prevailing understanding that it is highly efficient, while the second diet mentioned, in spite of a superficial equivalence to the diet of wheat and milk powder, was convincingly shown to be highly deficient.

The further discussion of the results of this experiment will be found following the description of the second experiment.

Experiment no. 2

The second program of experiments in this investigation is outlined in table 4, the general plan and objectives having been already explained.

When this program was outlined it was intended that the values for heat production from the two fasting periods would be averaged, to serve as a base value in computing the heat increments and net energy values of the diets for maintenance; and, similarly, that the results obtained in the two maintenance periods would serve as a basis for computing the heat increments and net energy values of the diets for growth.

TABLE 4

Experiment 2: Schedule of experimentation, representing average values per rat per day[1]

NUMBERS AND CHARACTER OF DIETS	PERIOD NOS.	PLANE OF NUTRITION	AGE OF RATS	WEIGHT OF RATS[2]	FRESH WEIGHT OF FOOD	DRY WEIGHT OF FOOD	NITROGEN OF FOOD	CARBON OF FOOD	ENERGY OF FOOD
			weeks	*gm.*	*gm.*	*gm.*	*mg.*	*gm.*	*Cal.*
1									
Wheat, milk powder	3	Growth	10	129	7	6.441	195	3.079	32.42
	4	Maintenance	12	133	5	4.601	140	2.200	23.16
	5	Fast	12	134	0	0	0	0	0
2									
Bread, butter, beef, roots	3	Growth	10	143	7	6.661	192	3.123	32.21
	4	Maintenance	12	149	5	4.758	137	2.231	23.01
	5	Fast	12	151	0	0	0	0	0
3									
Bread, butter, beef, green vegetables	3	Growth	10	140	7	6.703	191	3.063	32.28
	4	Maintenance	12	146	5	4.788	137	2.188	23.06
	5	Fast	12	147	0	0	0	0	0
4									
Bread, butter, beef, fruits	3	Growth	10	142	7	6.722	197	3.123	32.34
	4	Maintenance	12	145	5	4.802	141	2.231	23.10
	5	Fast	12	148	0	0	0	0	0
5									
Peanuts, fruits, green vegetables	3	Growth	10	116	7	6.769	207	3.128	32.44
	4	Maintenance	12	119	5	4.835	148	2.234	23.17
	5	Fast	12	120	0	0	0	0	0
6									
Corn meal, beans, cracklings, molasses	3	Growth	10	136	7	6.403	198	3.071	32.87
	4	Maintenance	12	139	5	4.574	142	2.194	23.48
	5	Fast	12	139	0	0	0	0	0

[1] All values are averages of results obtained with six animals.

[2] In fasting periods the live weights were taken 24 hours after the last feed was given; in other periods the weights are averages of weights taken at the beginning and end of respiration periods.

After the feeding and metabolism tests were finished, however, it was found that the plan of experimentation had miscarried in one important particular—that the rats had not been subjected to the 6-day feeding at the maintenance plane of nutrition, preliminary to the first determination of the heat production of fast, but that this fast had followed feeding at a rate so much in excess of maintenance that the fasting heat production generally exceeded that of the following maintenance period, the heat increments being negative quantities, with four rats among the six.

The first fasting heat measurements (period 1) for each group of treatments, therefore, were discarded as invalid, and the heat increments of maintenance were based on the second fasting heat measurements (period 5) alone. This incident serves the important purpose of emphasizing the significance of the plane of nutrition preceding the measurement of fasting katabolism, as affecting the magnitude of such measurements.

In recognition of the fact that the habit of an animal, with reference to heat production, as determined by the plane of nutrition to which it is accustomed, prominently affects metabolism during a subsequent fasting period, Forbes, Braman and Kriss ('30) recommended that feeding on the plane of energy equilibrium be followed as a standard treatment preliminary to the determination of the heat production of fast.

Having discarded the results of period 1, with each diet, therefore, there remained the question as to whether to base the computation of the heat increment of the food for growth (period 3) on an average of the results obtained in the two maintenance periods (periods 2 and 4), or on the second (period 4) alone.

While these two values were, presumably, equally valid, it was decided to use the second (period 4) alone, since the live weights in periods 3, 4 and 5 differed but little, thus requiring only small corrections of the heat production in computing to correspond to an average live weight, while a much

larger correction would have been necessary in the results from period 2, the first maintenance period, if these had been used.

On this account the results of experiment 2 depend on periods 3, 4 and 5 alone. Accordingly it is suggested that, in the use of the procedure described, for the evaluation of diets, the experimental subjects need be given only the three treatments—growth, maintenance, and fast—in the order named, which provides the proper preliminary treatment for the fasting heat measurement.

The average daily heat production per rat is given in table 5, computed to the basis of the average empty live weight, that is, the weight of the animal minus the contents of the alimentary tract.

These empty live weights were determined, for each diet, by means of a specially selected, representative group of rats, which were subjected to a feeding experiment for this purpose alone.

The correction of the heat production was made, in each case, in accord with the 0.73 power of the live weight, with the heat production of fast as the basis of correction.

The main results of the experiment are given in table 6, the upper half of the table representing the utilization of the diets for maintenance (period 4), and the lower half for growth (period 3).

The metabolizable energy which was computed as the gross energy minus the energy of the feces and urine, with a correction for the non-metabolizable fraction of the body gain, was unaffected by the plane of nutrition; but, with the two groups (maintenance and growth), each diet was characterized by an individual metabolizability.

In this relation the diets of special interest are no. 6 (corn meal, beans, cracklings, molasses), because of its maximum metabolizable energy value, no. 5 (peanuts, fruits, green vegetables), because of its minimum value, and no. 4 (bread, butter, beef, fruits) because it had a distinctly lower metabolizability than diets nos. 2 and 3, which differed from no. 4 only in the character of the vegetable component.

As compared with roots and green vegetables, fruits exercised a slight influence to lower the metabolizability of the diet in which it was contained.

The maximum metabolizability of diet no. 6 may be considered to be due especially to the high digestibility of the

TABLE 5

Experiment 2: Average daily heat production per rat

NUMBER AND CHARACTER OF DIETS	PERIOD NO.	PLANE OF NUTRITION	EMPTY LIVE WEIGHT	HEAT PRODUCTION	FASTING KATABOLISM [1]	CORRECTED HEAT PRODUCTION [1]
			gm.	*Cal.*	*Cal.*	*Cal.*
1						
Wheat, milk	3	Growth	122	22.30		23.36
powder	4	Maintenance	129	19.14		19.46
	5	Fast	130	18.78	18.99	18.99
2						
Bread, butter,	3	Growth	138	22.98		22.43
beef, roots	4	Maintenance	146	20.87		19.60
	5	Fast	148	18.09	16.64	16.64
3						
Bread, butter,	3	Growth	136	23.18		22.80
beef, green	4	Maintenance	142	21.97		21.02
vegetables	5	Fast	143	18.48	17.43	17.43
4						
Bread, butter,	3	Growth	137	22.02		21.58
beef, fruits	4	Maintenance	139	19.97		19.36
	5	Fast	142	16.80	15.93	15.93
5						
Peanuts, fruits	3	Growth	102	19.04		21.86
green	4	Maintenance	111	16.86		18.82
vegetables	5	Fast	112	14.59	16.45	16.45
6						
Corn, meal,	3	Growth	132	23.82		23.82
beans, crack-	4	Maintenance	136	22.43		22.04
lings, molasses	5	Fast	136	18.27	17.88	17.88

[1] Corrected to represent the average empty live weight of 132 gm.

sugar of the molasses, and to the fat of the corn meal and cracklings; and the minimum metabolizability of diet no. 5, to the fact that it contained no highly digestible component.

There was a difference of 27.3% between the metabolizable energy values of diets nos. 5 and 6, based on the value of no. 5.

TABLE 6

Experiment 2: Metabolizable energy, heat increments and net energy of diets for maintenance and for growth

NUMBERS AND CHARACTER OF DIETS	PLANE OF NUTRITION	FOOD, DRY MATTER	METABOLIZABLE ENERGY PER GRAM D. M.	HEAT PRODUCTION [1]	HEAT INCREMENT PER GRAM D. M.	NET ENERGY PER GRAM DRY MATTER
		gm.	*Cal.*	*Cal.*	*Cal.*	*Cal.*
1 Wheat, milk powder	Maintenance	4.601	4.30	19.46	0.10	4.20
2 Bread, butter, beef, roots	Maintenance	4.758	4.25	19.60	0.62	3.63
3 Bread, butter beef, green vegetables	Maintenance	4.788	4.29	21.02	0.75	3.54
4 Bread, butter, beef, fruits	Maintenance	4.802	4.12	19.36	0.71	3.41
5 Peanuts, fruits, green vegetables	Maintenance	4.835	3.62	18.82	0.49	3.13
6 Corn meal, beans, cracklings, molasses	Maintenance	4.574	4.52	22.04	0.91	3.61
1 Wheat, milk powder	Growth	6.441	4.30	23.36	2.12	2.18
2 Bread, butter, beef, roots	Growth	6.661	4.24	22.43	1.49	2.75
3 Bread, butter, beef, green vegetables	Growth	6.703	4.31	22.80	0.93	3.38
4 Bread, butter, beef, fruits	Growth	6.722	4.15	21.58	1.16	2.99
5 Peanuts, fruits, green vegetables	Growth	6.769	3.55	21.86	1.57	1.98
6 Corn meal, beans, cracklings, molasses	Growth	6.403	4.52	23.82	0.97	3.55

[1] Computed to the basis of the average empty live weight of 132 gm.

The values for heat production corresponded, in a general way, to the metabolizable energy, and seem not to be especially noteworthy.

The energy balances during feeding on the maintenance plane varied between —1.57 and + 0.82 Cal., and the values for heat production, as computed to the basis of the average empty live weight of the rats, were reasonably concordant. The heat increments for maintenance, therefore, rest on a fairly satisfactory basis.

The heat increment values of the diets for growth are in certain respects noteworthy. Thus, the increment of diet no. 1 (wheat, milk powder) is much higher than that of any other; and the increments of diets nos. 3 (bread, butter, beef, green vegetables) and no. 6 (corn meal, beans, cracklings, molasses) are the lowest of all.

Corresponding to the lowest heat increment for maintenance (diet no. 1; wheat, milk powder) is the highest net energy for maintenance. The net energy, for maintenance, of diet no. 5 (peanuts, fruits, green vegetables) was distinctly low; while diets nos. 2, 3, 4 and 6 had nearly the same net energy for maintenance.

The net energy value for each diet was consistently lower for growth than for maintenance.

Leaving out diets nos. 6 and 2, the net energy of the diets for growth, and the nitrogen retention, and the ratio of protein to non-protein energy stored, were all in the same order, that is, in order of ascending values—diets nos. 5, 1, 4, 3.

The fact that diet no. 6 was characterized by the maximum net energy value for growth appears to have been due to the same circumstances that gave to this diet its maximum metabolizable energy value, that is, the high sugar content of the molasses, and the relatively high fat contents of the corn meal and the cracklings; and, similarly, the fact that diet no. 5 was characterized by the minimum net energy value for growth seems to have been due to the same fact that gave this diet its minimum metabolizable energy value, that it contained no highly digestible component.

Aside from these two values the most notable is the low net energy of diet no. 1 (wheat, milk powder) for growth. This is most readily understandable on the assumption (for which, however, there is no warrant except hypothesis) that the protein value was injured during the drying of this particular lot of milk.

It is necessary not to be misled by high net energy values for body increase of diets which, in view of their composition, would be considered poor foods for growth, since such diets may not be efficient for the production of protein increase. In other words, net energy is not a measure of protein value.

The digestibility of the experimental diets appears not to have been affected by the plane of nutrition in any generally significant manner. The outstanding facts observable in the data, as presented in table 7, are that diet no. 5, of peanuts, fruits and green vegetables, was much lower in digestibility of both energy and nitrogen, at both planes of nutrition, than was any other; that the digestibility of the energy of diet no. 3, of bread, butter, beef and green vegetables, was higher than that of any other, at both planes of nutrition; and that the digestibility of the nitrogen of diet no. 1, of wheat and milk powder, and no. 3, of bread, butter, beef and green vegetables, was appreciably higher, at both planes of nutrition, than was that of any others.

In consideration of gross efficiency—based upon total intake rather than intake in excess of the maintenance requirement—the quantities of nitrogen and energy retained by the animals on diets approximating the maintenance level show that this group of diets were more favorable to nitrogen than to energy retention. Diet no. 1, of wheat and milk powder, however, was lowest in nitrogen storage at the lower plane, and next to the lowest at the higher plane, which suggests again that the protein value of the particular lot of milk powder used in experiment no. 2 had been damaged during drying.

At the higher plane of intake there was 70% difference between the largest and the smallest quantities of nitrogen stored from these diets (nos. 5 and 2, respectively); also the

TABLE 7

Experiment 2: Average digestibility and daily retention of energy and nitrogen of diets

DIET NO.	CHARACTER OF DIET	PLANE OF FOOD INTAKE	ENERGY			NITROGEN			RATIO OF PROTEIN TO NON-PROTEIN ENERGY RETAINED
			Food	Digested	Retained	Food	Digested	Retained	
			Cal.	%	*Cal.*	*mg.*	%	*mg.*	
1	Wheat, milk powder	Maintenance	23.16	91.8	0.79	140	89.3	17	
2	Bread, butter, beef, roots	Maintenance	23.01	93.2	— 0.38	137	84.7	33	
3	Bread, butter, beef, green vegetables	Maintenance	23.06	94.3	— 1.15	137	89.1	35	
4	Bread, butter, beef, fruits	Maintenance	23.10	91.0	0	141	82.3	25	
5	Peanuts, fruits, green vegetables	Maintenance	23.17	82.3	0.82	148	72.3	23	
6	Corn meal, beans, cracklings, molasses	Maintenance	23.48	92.8	— 1.57	142	83.8	26	
1	Wheat, milk powder	Growth	32.42	91.7	5.82	195	89.2	60	0.54
2	Bread, butter, beef, roots	Growth	32.21	92.7	5.87	192	82.8	80	0.88
3	Bread, butter, beef, green vegetables	Growth	32.28	94.3	6.28	191	88.5	77	0.72
4	Bread, butter, beef, fruits	Growth	32.34	91.7	6.43	197	84.8	71	0.61
5	Peanuts, fruits, green vegetables	Growth	32.44	80.4	5.36	207	72.0	47	0.43
6	Corn meal, beans, cracklings, molasses	Growth	32.87	92.8	5.58	198	83.3	65	0.66

nitrogen retention from diet no. 6 of corn meal, beans, cracklings and molasses, was surprisingly efficient.

Among the three bread, butter and beef diets (nos. 2, 3 and 4), the proportion of protein to non-protein energy stored was favored first by the roots, second by the green vegetables, and third by the fruits.

The energy retention at the lower plane of nutrition was at or near zero, as intended, while at the higher plane the storage of energy did not differ greatly in accord with the nature of the diets. At the higher plane, the diet which caused the largest energy storage (no. 4) expressed a 20% greater gross energy efficiency than did the diet (no. 5) from which the smallest quantity of energy was stored.

The ratios of protein to non-protein energy retained suggest, as did the net energy values, that in diet no. 1 (wheat and milk powder) the milk protein was 'below par'; though the protein value was lowest of all in diet no. 5, of peanuts, fruits and green vegetables.

The wheat used in diet no. 1, in both experiments, was of the same lot, but the milk powder in the two experiments differed as to trade name. The question as to the cause of the difference in the nutritive value of diet no. 1, as made with the two lots of milk powder, and as used in the two experiments, recalls the findings of Mitchell and Fairbanks ('35) that the cystine content of milk may be diminished by excessive heat, in drying on a drum; of Sharpenak and Eremin ('35) that the 'total albumin' of milk contains more than three times as much methionine as cystine; and of Rose ('37) that methionine is an essential amino acid, while cystine is not essential.

DISCUSSION

In the first experiment, the demonstrated method of comparison of nutritive values of diets, by means of a series of carbon and nitrogen balances determined in the course of a growth experiment with constant food intake, constitutes a highly critical and significant basis for the comparison of the efficiency of diets for purposes of growth.

This method is noteworthy on account of the facts that it demonstrates the developing effects of dietary differences and deficiencies through comparatively long periods of time, the constant level of food intake leading to increasingly severe nutritive conditions; and also that it avoids certain incompletely justified assumptions involved in net energy determinations.

The results obtained by this method lend themselves especially to expression as 1) the metabolizable energy and the heat production in relation to the food energy. 2) The gain in weight before the limit of such increase is reached. 3) The gains of energy and of protein in relation to food energy and protein.

This procedure, therefore, is well adapted to the comparison of diets; but, because of the fact that it expresses the gross efficiency, instead of the net efficiency point of view—there being no separation of nutriment used for maintenance from nutriment used for gain, it is not adapted to the measurement of nutritive values in terms of units.

The second experiment, conducted in harmony with the net energy conception, entails all of the advantages, and the embarrassments, of this superficially logical, but fundamentally imperfect point of view.

The net energy method leads to measurements in terms of calories, of the values of foods as sources of energy; but this is by virtue of a comparison of results obtained from the feeding of two rations—a basal ration, and another which is the same plus a known quantity of the foodstuff to be evaluated; and by virtue of an assumption that the energy expenses and losses of utilization of the smaller or basal ration are the same when this ration is fed as a part of the supplemented ration as when it is fed alone.

This assumption is superficially logical, but it is not exactly physiological.

Also, the net energy method assumes that the energy expense of food utilization is proportional to the quantity of food eaten, which is not exactly true.

Further, net energy values are lacking in perfection, as practical constants, by virtue of the fact that they are prominently affected by differences in environmental conditions which are frequently encountered.

It is true, however, that despite these imperfections, the method of measurement of food values in terms of net energy has much in its favor, especially for limited purposes of close discrimination, under rigorously controlled conditions of experimentation.

While a basis does not exist for a close comparison of the two methods demonstrated, for the study of the nutritive values of foods, the results are in general harmony, insofar as they are comparable.

SUMMARY

New equipment and two experimental procedures were demonstrated, with growing albino rats as subjects, for the comparison and the measurement of the nutritive values of human diets.

The equipment was a series of water-sealed respiration chambers, for the determination of the quantitative and the qualitative character of metabolism, by means of the carbon and nitrogen balance method, under normal conditions of physical activity of the subjects.

One of the procedures consisted of growth experiments, in the course of which were determined series of carbon and nitrogen balances, at intervals, with the same food intake throughout, for the purpose of observing the character of the metabolism as affected by the progressively increasing severity of the nutritive conditions imposed by the gain in weight of the subjects.

This procedure yielded results in terms of gross efficiency, covering requirements for maintenance and production together.

The second procedure consisted of nutritive measurements, also by means of carbon and nitrogen balances, representing the net energy point of view. Balances were determined

during 1) fast, 2) feeding at the maintenance level, and 3) feeding at a higher plane; and upon these balances were based values for heat increment and net energy, separately for maintenance and for growth.

The two distinctly different methods of experimental management afforded equally distinct means of comparison and measurement of the values of diets as sources of nutritive energy and protein. The respective advantages and limitations of the two procedures are pointed out.

In the comparison of the nutritive values of diets the results obtained by the two procedures were in harmony, insofar as they were comparable.

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FURTHER STUDIES ON THE UNSATURATED FATTY ACIDS ESSENTIAL IN NUTRITION [1]

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SIX FIGURES

(Received for publication October 16, 1937)

The earlier work by Evans and Burr ('27 a, b and '28), by Burr and Burr ('29 and '30), and by Evans and Lepkovsky ('32 a) has clearly established the fact that the rat does not thrive on diets rigidly devoid of fat, but develops a characteristic deficiency disease. The most important 'fat deficiency' symptoms are: a retardation and gradually a complete cessation of growth, scaly condition of skin, renal lesions often manifesting themselves in the appearance of blood in the urine, abnormally high water consumption, and irregularities of ovulation. Gestation, lactation, and male reproductive functions are also considerably impaired (Evans, Lepkovsky and Murphy, '34 a, b). The need of the animal body is not, however, as the above mentioned investigators have demonstrated, for fat in general, but for certain unsaturated fatty acids. The ineffectiveness of saturated fatty acids was shown in a striking manner by Evans and Lepkovsky ('32 b), who fed several preparations of these acids as the sole source of energy (about 60% of the diet) and found that rats on such diets developed a condition similar to the 'fat deficiency.'

[1] Aided by grants from the Board of Research and the College of Agriculture of the University of California and the Rockefeller Foundation of New York.

[2] Holder of a Finnish State Fellowship for the most part of the work reported here; during the last phase of the work, a Rockefeller Foundation Fellow.

There are also considerable differences between the unsaturated fatty acids in their capacity to cure the 'fat deficiency' syndrome. Only two acids, namely linoleic (Burr and Burr, '29) and linolenic (Burr, Burr and Miller, '32) have so far been found effective, whereas oleic (Evans and Lepkovsky, '32 a) and alpha-eleostearic (Burr, Burr and Miller, '32) acids have proved ineffective. Arachidonic acid was also tested by Burr, Burr and Miller, but the result did not seem quite convincing. It was found that an addition of methyl arachidonate as 10% of a mixture of equal parts of the methyl esters of linoleic and linolenic acids slightly decreased the curative potency of the original mixture.

In the beginning of this work the above mentioned fatty acids were the only ones tested. It seemed highly desirable to gather some more information in this field by testing as many new fatty acids as possible and, so doing, perhaps to reach gradually some general conclusions as to why certain fatty acids are capable of curing the 'fat deficiency' syndrome while others are not. During the work reported here the following fatty acids not before tested have been examined with regard to their effectiveness in curing the 'fat deficiency:' erucic, $\triangle^{12:13}$-oleic, ricinoleic and chaulmoogric. In addition, arachidonic acid was retested, and the effect of different levels of linoleic acid was measured somewhat more accurately than earlier. Also the alcohol corresponding to linoleic acid, linoleyl alcohol, was tested.

EXPERIMENTAL

Preparation of the materials tested. The fatty acids were fed in the form of the methyl or ethyl ester. As Lepkovsky, Ouer and Evans ('35) have shown, these esters are utilized by the rat even at comparatively high levels with almost the same ease as the glycerides. In the course of this work the following substances were tested: methyl linoleate, methyl arachidonate, ethyl eruceate, ethyl $\triangle^{12:13}$-oleate, methyl ricinoleate, ethyl chaulmoograte and linoleyl alcohol.

Methyl linoleate was prepared from corn oil according to the bromination method of Rollet ('09). The resulting tetrabromostearic acid was recrystallized several times from high-boiling petroleum ether, until it was snow-white and melted sharply at 114.5 to 115°. The debromination was carried out exactly according to the original method, and the methyl ester was distilled under highly reduced pressure (1 to 2 mm.). The final product had an iodine number of 168 and a mean molecular weight (by saponification) of 291 (theory 172.5 and 294, respectively).

Methyl arachidonate was a gift from Prof. J. B. Brown, of the Ohio State University, and Mr. George Y. Shinowara associated with him. According to them it had an iodine number 315 (Wijs 2 hours) and a mean molecular weight 314.8 (theory 319 and 318, respectively).

Erucic acid was isolated from rape seed oil according to Noller and Talbot ('30). The acid (m.p. 33° to 34°) was esterified with absolute ethyl alcohol in the presence of concentrated sulfuric acid, and the ester was distilled in high vacuum. The final product, ethyl eruceate, had an iodine number of 68.5 and a mean molecular weight of 364 (theory 69.3 and 366, respectively).

Ricinoleic acid was prepared from castor oil principally by the method of Rider ('31). The acid was converted into the methyl ester by refluxing it with methanolic hydrochloric acid, and the ester was distilled in high vacuum. The resulting methyl ricinoleate had an iodine number of 78.9 and a mean molecular weight 310 (theory 81.3 and 312, respectively).

The ethyl ester of $\triangle^{12:13}$-oleic acid may be prepared, as Grün and Czerny ('26) have shown, from the corresponding ester of 12-hydroxystearic acid. If the latter is heated to 220° with 2% of beta-naphthalene sulfonic acid, water is split off and a double bond is formed in the position 12:13. This reaction runs very smoothly and is almost quantitative. Methyl acetylricinoleate was the starting material for the preparation of the ethyl 12-hydroxystearate; it had been prepared in the course of the isolation of ricinoleic acid from

castor oil. This substance was first hydrogenated at 100° and atmospheric pressure with Raney nickel as a catalyst. The Raney metal was activated according to Covert and Adkins ('32). The resulting methyl acetoxystearate was then saponified and reesterified with ethanolic hydrochloric acid. This gave us ethyl 12-hydroxystearate, which after two recrystallizations from acetone (m.p. was then 51.5 to 52°) was treated with beta-naphthalene sulfonic acid according to the method of Grün and Czerny. The resulting ethyl $\Delta^{12:13}$-oleate is, as these authors have demonstrated, a mixture of about one-third of cis-form and two-thirds of trans-form. A part of this mixture was used as such for the feeding experiments, but in view of the fact that as far as known all naturally occurring fatty acids have the cis-configuration, it was felt desirable also to test the biological activity of the isolated cis-form. Therefore the ester was saponified, and the two isomeric forms were separated by a method described in the work of Grün and Czerny. For our purpose it was not, however, necessary to carry the purification quite so far as these workers did; so the isolated cis-acid contained still some of the trans-form and melted at 11 to 13° (instead of 10°). For feeding tests this acid was esterified with absolute ethyl alcohol and concentrated sulfuric acid. The characteristics of our preparations of ethyl $\Delta^{12:13}$-oleate were: cis-trans mixture: iodine number 81.7, mean molecular weight 309; cis-form: iodine number 80.9, mean molecular weight 305 (theory 81.8 and 310, respectively).

Ethyl chaulmoograte was kindly furnished by Prof. C. D. Leake of the Medical School of the University of California. The sample was not large enough to permit any chemical tests.

Linoleyl alcohol, a compound which evidently has never before been synthesized, was prepared from methyl linoleate by the reduction method of Bouveault and Blanc ('03). The instructions given by Reid and collaborators ('35) for the preparation of oleyl alcohol were followed in their main points. The yield was good and the product was apparently of high degree of purity. A more complete account of the

preparation and properties of linoleyl alcohol has been given elsewhere (Turpeinen, '38).

Preparation of animals. The methods used were principally the same as those employed earlier in this laboratory by Evans and Lepkovsky ('32 a). Female rats were used throughout. They were weaned on the twenty-first day of life and placed on the following fat-low diet:

Diet 667

Casein (gasoline-washed)	24
Sucrose	72
Salts (McCollum 185)	4

This diet was supplemented with 1) 1.0 gm. of ether-extracted brewer's yeast six times weekly, 2) the non-saponifiable fraction of cod liver oil equivalent to 80 mg. of the original oil six times weekly, and 3) the non-saponifiable fraction of wheat germ oil equivalent to 500 mg. weekly. The positive controls received in addition 60 mg. of an ethyl linoleate preparation (approximately 80% pure) six times weekly.

The non-saponifiable fractions of C.L.O. and W.G.O. were prepared by refluxing the oils with a slight excess of 20% methanolic potassium hydroxide under hydrogen for 1 hour, by diluting the mixture with about twice its volume of distilled water, and by taking the non-saponifiable matter into peroxide-free ether.

The animals were kept three in a cage on wire screens. They were weighed at 5-day intervals, and the incidence of estrus was observed by making vaginal smears daily. The growth of the 'fat-deficient' animals (eighty-two rats) as well as that of positive controls (eight rats) is shown in figure 1. The animals on the fat-low diet plateaued in this case at about 160 gm. The positive controls grew much better, but even they did not quite equal in growth the animals on our stock diets. This was probably due to the inadequacy of the ethyl linoleate dose, for the experiments which will be presented below have demonstrated that about 100 mg. of methyl linoleate daily is needed to produce a maximal renewed growth in 'fat-deficient' female rats. The ovulation of the

'fat-deficient' animals was considerably disturbed, although in this respect there was a great deal of variation among individuals. While a few animals had a normal estrous rhythm,

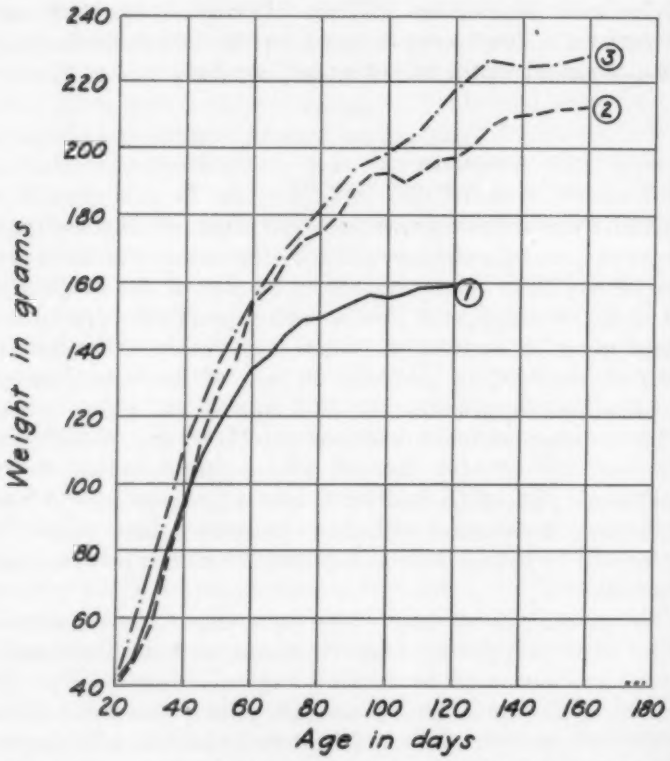

Fig. 1 Growth of female rats on (1) fat-free diet, (2) the same diet with 50 mg. of ethyl linoleate daily, (3) stock diet XIV.

some did not ovulate at all. The positive controls showed a normal estrous rhythm. The ovulation data are given in table 1.

The skin symptoms of the 'fat-deficient' animals were, as they have always been in this laboratory (compare Evans and Lepkovsky, '32 a), comparatively mild. Only some scaliness and dandruff, and a tendency to lose hair on the face, particularly around the eyes, were observed. Several cases of hematuria were noticed, and the increased consumption of water was also evident, although no quantitative measurements were done.

Conduct of the tests. The animals which had reached a growth plateau were employed as test animals after they had shown a constant (within small fluctuations) or slightly declining weight over a period of at least 30 days. In the

TABLE 1

Incidence of estrus

	Fat-low group (82 animals)	*Positive controls (8 animals)*
Average number of vaginal cycles up to 120 days of age	5.8	14.0
Number of animals having the following number of cycles up to 120 days of age		
13–16 cycles	5	7
9–12 cycles	17	1
5– 8 cycles	26	0
0– 4 cycles	34	0

beginning of the tests the animals were on the average 156 days old (120 to 185 days) and weighed on the average 171 gm. (128 to 208 gm.). The test materials were fed together with the vitamin supplements on separate dishes daily (except Sundays) and were usually quickly consumed. Methyl ricinoleate and ethyl chaulmoograte, which rats refused to eat, were given with a small pipette directly into the mouth. A group of three rats was employed for each test. The curative effect was measured mainly by the intensity of the renewed growth, for this was easiest to record quantitatively, although the data concerning ovulation often gave valuable additional insight into the potency of the test substances. The disappearance of hematuria was also a good criterion of the effectiveness of a substance, but because only one-sixth

of the animals had exhibited a macroscopically noticeable hematuria before the tests, the occurrence of this symptom was too rare to have any practical significance. The test period lasted 50 days; the gain in weight and the number of vaginal cycles during this period were recorded, and the latter was compared with the number of cycles during an equally long period immediately preceding the test.

RESULTS AND CONCLUSIONS

The results of the tests as far as the growth response is concerned may be easily seen by inspecting the figures 2 to 6. These results are summarized in table 2 below, in which also the data concerning ovulation are given.

TABLE 2

Effects of the test substances on 'fat-deficient' rats

SUBSTANCE	DAILY DOSE	AVERAGE GAIN IN WEIGHT IN 50 DAYS (WITH PROBABLE ERROR)	AVERAGE NUMBER OF ESTROUS CYCLES DURING 50-DAY PERIOD		CONCLUSION
			Before test	During test	
	mg.	*gm.*			
Methyl linoleate	200	41.3 (± 7.4)	5.0	9.7	Producing maximal effect
Methyl linoleate	100	45.7 (± 3.0)	5.7	10.7	Producing maximal effect
Methyl linoleate	50	29.5 (± 4.5)	5.3	9.3	Effective
Methyl linoleate	25	20.3 (± 1.6)	5.3	8.0	Effective
Ethyl 12: 13-oleate, cis-trans	100	0.0 (± 2.7)	6.3	5.7	Ineffective
Ethyl 12: 13-oleate, cis	100	— 2.3 (± 2.4)	4.0	4.7	Ineffective
Methyl ricinoleate	100	— 10.3 (± 4.1)	3.0	1.0	Ineffective
Ethyl erucoate	100	2.7 (± 0.6)	7.7	6.0	Ineffective
Ethyl chaulmoograte	100	Animals died	—	—	Toxic
Ethyl chaulmoograte	50	— 2.3 (± 1.2)	9.3	5.7	Ineffective
Methyl arachidonate	100	43.7 (± 3.6)	2.3	7.7	Producing maximal effect
Methyl arachidonate	33	44.0 (± 4.3)	0.7	7.0	Producing maximal effect
Linoleyl alcohol	100	23.7 (± 0.2)	8.3	9.7	Effective

The experiments with methyl linoleate were done mainly in order to find the minimal dose giving the maximal growth response, thus furnishing an idea of quantitative effectiveness of this substance. Furthermore, it was hoped that these experiments would guide us in the matter of the dosage with other fatty acid preparations. It is, of course, evident that

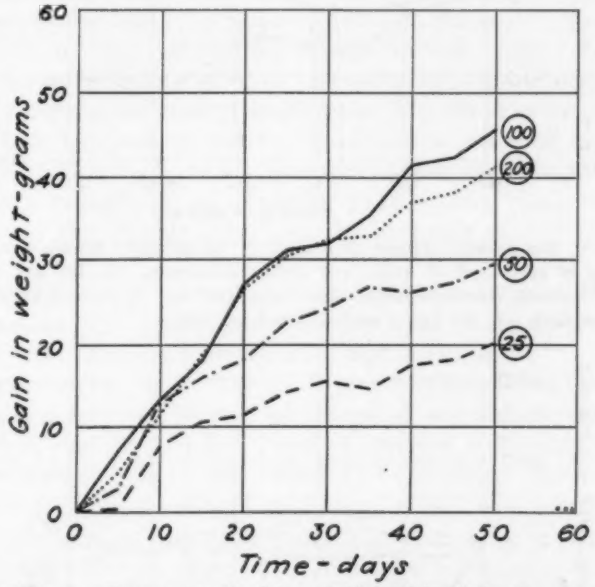

Fig. 2 The growth response of plateaued 'fat-deficient' female rats to the feeding of methyl linoleate. (The daily dose is given at the end of each curve.)

these results cannot claim a high degree of accuracy in the quantitative sense, because the groups of test animals were small, and, consequently, the probable errors of the means are comparatively large. It seems justified, however, to conclude from these experiments that about 100 mg. of methyl linoleate is needed to produce maximal growth in 'fat-deficient' female rats. The correct value is certainly between 50 mg. and 100 mg. and very probably closer to the latter.

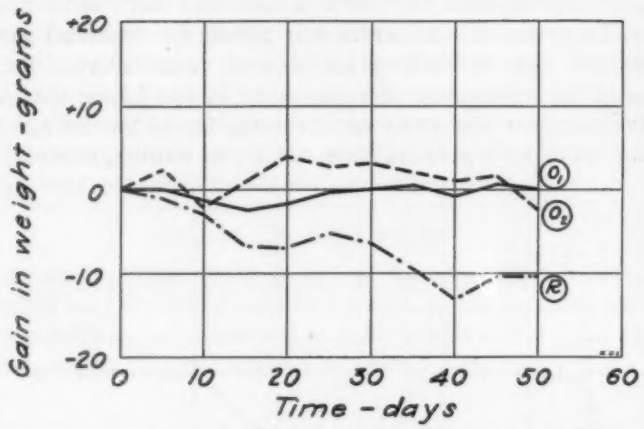

Fig. 3 The growth response of plateaued 'fat-deficient' female rats to the feeding of ethyl $\Delta^{12:13}$-oleate and methyl ricinoleate. O_1, 100 mg. of ethyl $\Delta^{12:13}$-oleate, cis-trans-mixture, daily. O_2, 100 mg. of ethyl $\Delta^{12:13}$-oleate, cis-form, daily. R, 100 mg. of methyl ricinoleate, daily.

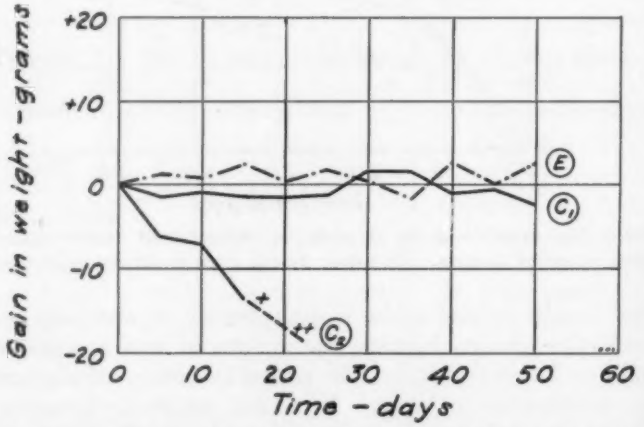

Fig. 4 The growth response of plateaued 'fat-deficient' female rats to the feeding of ethyl eruceate and ethyl chaulmoograte. E, 100 mg. of ethyl eruceate daily; C_1, 50 mg. ethyl chaulmoograte daily; C_2, 100 mg. ethyl chaulmoograte daily; + denotes the death of a test animal.

$\Delta^{12:13}$-oleic acid ($\Delta^{12:13}$-octadecenoic acid) was regarded as being particularly worth testing, because its only double bond is in the same position as the second double bond of linoleic acid (which structurally is $\Delta^{9:10,12:13}$-octadecadienoic acid). It did not seem wholly improbable that the animal body, which is known to be capable of synthesizing ordinary oleic acid ($\Delta^{9:10}$-octadecenoic acid), could also be capable of introducing the 9:10 double bond into the molecule of the $\Delta^{12:13}$-acid, thus converting it into linoleic acid. In this case the $\Delta^{12:13}$-oleic acid, of course, should be effective in curing the 'fat-deficiency' syndrome. The ethyl ester of this acid was, however, found to be without a trace of activity (fig. 3). This leads to the conclusion that the animal body, although capable of forming the 9:10 double bond, cannot introduce this double bond into the molecule of $\Delta^{12:13}$-oleic acid.

Ricinoleic acid (12-hydroxy-$\Delta^{9:10}$-octadecenoic acid) and erucic acid ($\Delta^{13:14}$-docosenoic acid) were found to be completely devoid of curative effects (figs. 3 and 4).

Chaulmoogric acid (13-[$\Delta^{2:3}$-cyclopentenyl] tridecanoic acid) at a level of 50 mg. of the ethyl ester daily was ineffective, and at 100 mg. level it showed a marked toxic action, killing the animals within 17 to 22 days. In view of the data of Emerson and Anderson ('34), who state that 30 cc. of ethyl chaulmoograte per kilogram when given as a single subcutaneous dose still represents a tolerated dosage, it would seem that in our experiments the substance displayed an unexpectedly high degree of toxicity. It is easy to understand, however, that the sensibility of 'fat-deficient' rats to chaulmoogric acid, a substance capable of causing renal irritation, may be considerably increased on account of the inferior condition of their kidneys. In fact, all our rats receiving the larger dose of ethyl chaulmoograte developed a severe hematuria during the tests.

The tests with arachidonic acid (an eicosatetrenoic acid, in which the positions of the double bonds are not definitely known) led to most interesting results (fig. 5). It proved

to be a very powerful agent in curing the 'fat-deficiency' syndrome. It was even more effective than linoleic acid. While about 100 mg. of methyl linoleate were needed to produce maximal growth in plateaued 'fat-deficient' animals, the same effect could be obtained with 33 mg. of methyl arachidonate. (The lag in the beginning of the 33 mg. curve

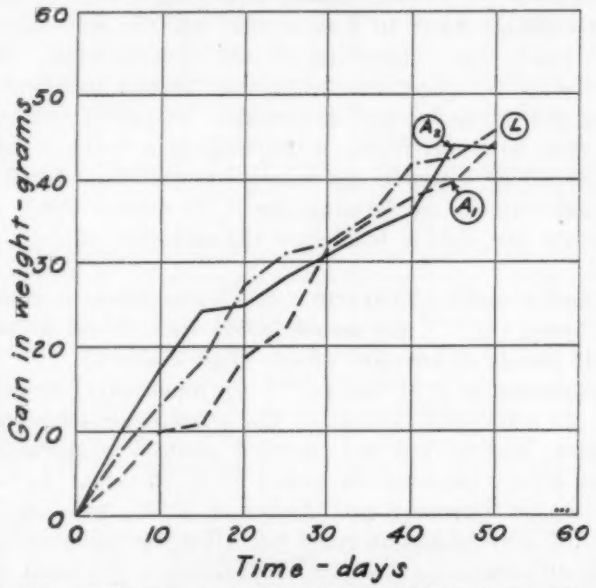

Fig. 5 The growth response of plateaued 'fat-deficient' female rats to the feeding of methyl arachidonate. A_1, 33 mg. of methyl arachidonate daily; A_2, 100 mg. of methyl arachidonate daily; L, 100 mg. of methyl linoleate daily—for comparison.

is probably not significant, because of the striking agreement of the curves in the later phases.) Whether even smaller doses of arachidonic acid would give maximal growth response must be left undecided. The tests reported here, however, justify the conclusion that arachidonic acid is at least approximately three times as effective as linoleic acid in curing the 'fat-deficiency' syndrome.

Linoleyl alcohol ($\triangle^{9:10,12:13}$-octadecadienol) was tested mainly in order to find out whether the presence of a carboxyl group was essential for the effectiveness of a substance as a curative agent. In our experiments linoleyl alcohol exhibited unmistakable curative properties, although it did not equal linoleic acid in this respect (fig. 6). The activity of linoleyl alcohol was approximately one-fourth to one-third of that of methyl linoleate. Now, it is possible that the difficulty of the absorption of linoleyl alcohol may account for a part of this

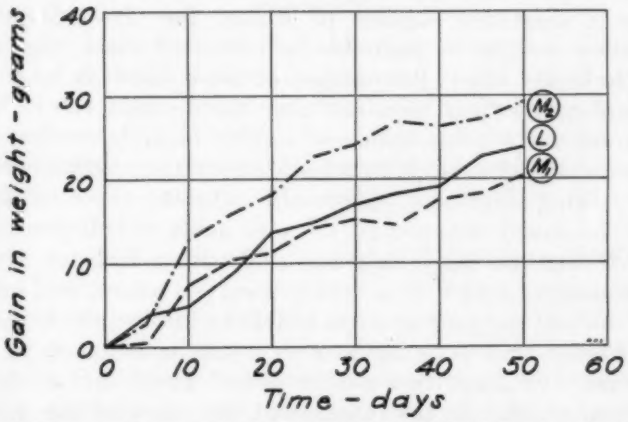

Fig. 6 The growth response of plateaued 'fat-deficient' female rats to the feeding of linoleyl alcohol (two levels of methyl linoleate included for comparison). L, 100 mg. of linoleyl alcohol daily. M_1, 25 mg. of methyl linoleate daily. M_2, 50 mg. of methyl linoleate daily.

difference. Channon and Collinson ('28) have demonstrated that the capacity of the rat to absorb higher alcohols like cetyl and oleyl alcohols is rather limited. In their experiments the absorption of oleyl alcohol by rats was on the average only 46 mg. daily. If this is also the case with linoleyl alcohol, the above results do not give a quantitatively correct picture of the effectiveness of this substance.

In conclusion it is perhaps worth while to summarize the evidence now available as to the curative effects of different

substances in the 'fat-deficiency' disease and to see if any general conclusions could be drawn. Including the fatty acids tested previously, we obtain the following classification:

Effective	*Ineffective*
Linoleic acid	Ordinary oleic acid
Linoleyl alcohol	$\Delta^{12:13}$ -oleic acid
Linolenic acid	Erucic acid
Arachidonic acid	Ricinoleic acid
	Chaulmoogric acid
	α-eleostearic acid

Now what are the particular features in the molecule which make a substance capable of curing the 'fat-deficiency?' Curative potency is undoubtedly linked in some way with double bonds. Is it the number of these which is most important or do their positions play the decisive role? Five different fatty acids with one double bond have thus far been tested and none of them has exhibited any activity whatever. They represent sufficiently different types of fatty acids (ordinary oleic and $\Delta^{12:13}$ -oleic acids with double bonds in different positions, ricinoleic acid with a hydroxy group, chaulmoogric acid with a cyclopentene structure, and erucic acid with 22 carbon atoms) to enable us to conclude with fair probability that fatty acids with a single double bond are inactive. At least two double bonds would appear to be necessary. But on the other hand, the need of the animal body cannot be simply for fatty acids with two or more double bonds, for α-eleostearic acid ($\Delta^{9:10,\,11:12,\,13:14}$-octadecatrienoic acid) is inactive. The double bonds must evidently occupy certain positions. Both linoleic and linolenic acids possess double bonds in the positions 9:10 and 12:13, the latter acid has an additional double bond at 15:16. The last-mentioned double bond cannot, however, be of any importance, for linoleic acid is already, as Burr, Burr and Miller showed, quite as effective as linolenic. Thus the double bonds 9:10 and 12:13 are apparently the features which are responsible for the curative properties of an 18 carbon atom fatty acid. The carboxyl group is not essential; it may be changed for an alcohol group, although with some loss of potency.

The superiority of arachidonic acid as a curative agent to any other substance thus far tested would suggest the tentative hypothesis that the need of the animal body might be primarily for this acid. The wide occurrence of this acid in various tissues as a component of physiologically important substances (phospholipids) would also support the notion of its indispensability. Linoleic acid and the other 'effective' substances could perhaps be beneficial solely on the account of their conversion into arachidonic acid in the body.

SUMMARY

1. The effectiveness of the following substances to cure the 'fat-deficiency' disease in rats was tested for the first time: erucic acid, ricinoleic acid, $\triangle^{12:13}$-oleic acid, chaulmoogric acid and linoleyl alcohol. Arachidonic acid was retested, and the effects of different levels of linoleic acid were estimated. The fatty acids were fed in the form of their methyl or ethyl esters.

2. Erucic, ricinoleic, $\triangle^{12:13}$-oleic, and chaulmoogric acids proved ineffective.

3. In experiments conducted with four levels of linoleic acid, it was found that the maximal growth response in plateaued 'fat-deficient' females ensued when approximately 100 mg. of methyl linoleate were fed daily; twice this amount did not further improve growth, whereas half of it was patently inadequate.

4. Linoleyl alcohol showed some curative properties, although it was less effective than linoleic acid.

5. Arachidonic acid was found to be a powerful curative agent; it produced maximal growth response when 33 mg. of the methyl ester were fed daily, and was hence very definitely superior to linoleic acid in this respect.

The author is deeply indebted to Prof. Herbert M. Evans for suggesting this problem, for the generous provision of facilities in his laboratory, and for his interest and help during the course of the work.

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THE INFLUENCE OF HYDROGENATION AND OF YEAST IN COUNTERACTING COD LIVER OIL INJURY IN HERBIVORA, AND THE INFLUENCE OF SALMON OIL ON MILK FAT SECRETION [1]

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ONE FIGURE

(Received for publication October 18, 1937)

In an earlier report (McCay and Maynard, '35) the effect of cod liver oil, shark liver oil and salmon oil upon the composition of blood and milk was discussed. From this earlier study, the possibility was suggested that the constituent of cod liver oil which produces the decrease in the milk fat percentage might be the same as that responsible for the development of muscle lesions in Herbivora.

Hilditch and Thompson ('36) found that feeding cod liver oil to a cow increased the secretion of the highly unsaturated fatty acids of the C20–22 series into the milk fat. Since cod liver oil is rich in this series and shark liver oil is poor, it might seem that these fractions might be responsible for producing the heart lesions in Herbivora, as well as decreasing the secretion of milk fat. On the other hand, salmon oil is rich in this same series of unsaturated acids but fails to react upon the lactating cow with the constancy of cod liver oil.

In our earlier studies it was found that the active factor or factors for the production of muscle lesions and for lowering the secretion of milk fat were found in the saponifiable fraction of cod liver oil. This suggested that the activity might be due to some of the unsaturated fatty acids. To test

[1] Reported before the Rochester meeting of the A.C.S., September, 1937.

this hypothesis further, hydrogenation was employed as a means of partly saturating the double bonds of these unsaturated acids.

HYDROGENATED COD LIVER OIL[3] AND MILK FAT SECRETION

In a preliminary trial six cows were divided into two groups of three each. The first group was fed cod liver oil at the rate of $\frac{1}{3}$ cc. per day per kilo of live weight, while the second group was fed a similar amount of cod liver oil hydrogenated to the point where it still melted below 53°C. This experiment included a preliminary period before the oil feeding and one following to determine the level of fat secreted by each cow before and after, as well as during the oil feeding.

The three cows fed the ordinary cod liver oil responded in the usual manner with a decrease in the percentage of fat in the milk and an increase in the iodine number of the milk fat. In the case of those fed the hydrogenated oil, there was no change in two cases, but a sharp drop during the second week of oil feeding in the case of the third cow. However, the milk fat percentage of this cow never recovered during the 3 weeks following the oil feeding. This phenomenon is contrary to that observed uniformly after feeding cod liver oil. Ordinarily in cod liver oil feeding the fat per cent starts to recover as soon as the oil feeding stops and is usually back to the normal level for the individual at the end of 2 or 3 weeks.

These findings indicated that the hydrogenation of cod liver oil altered it to the extent that it no longer affected the secretion of milk fat. A second experiment to test further the effect of hydrogenated cod liver oil upon the secretion of milk fat was then designed.

In this next study a large sample of cod liver oil was purchased in the market. Half of this was hydrogenated so that the iodine number fell from 160 to 69. The melting point of the hydrogenated oil was below 53°C. The vitamins were probably partly destroyed because of the high temperature

[3] The cod liver oil used in these studies was hydrogenated through the courtesy of H. G. Miller of the Procter and Gamble Company.

of the hydrogenation, but in the present case the oil was not employed for a vitamin supplement.

The purpose of these new experiments with this hydrogenated product was first to determine if hydrogenation altered the component responsible for reducing the milk fat in a lactating cow.

Numerous studies have shown that the response of the lactating cow to the feeding of cod liver oil is remarkably constant. If a cow is fed ½ cc. of cod liver oil per kilo of live weight per day, the decline in the percentage of milk fat usually begins within the first week and within 2 to 3 weeks the fat that is secreted into the milk is markedly below the normal for the individual and the basal ration. Because of the constancy of this reaction few test animals need be used.

For the present study six cows were so selected that none would be more than 6 months with calf at the end of the oil feeding. In order to avoid environmental factors, these were divided into two groups of three each. They were fed the regular winter ration for a period of 2 weeks in order to establish the fat percentage of the milk of each individual. The members of group 1 were then fed ½ cc. of cod liver oil per kilo of body weight daily while group 2 was fed the same amount of hydrogenated oil. This oil feeding was continued for 3 weeks. Both groups were then allowed a rest period of 3 weeks with no oil. The oil feeding was then reversed so that group 1 was fed the hydrogenated product and group 2 the original oil. After this second period of oil feeding for 3 weeks, the cows were turned to pasture with no further oil feeding. An aliquot of milk was taken at each milking. The fat was determined at weekly intervals upon these pooled aliquots by the Roese-Gottlieb method. The iodine number was run by the Hanus method.

The results of this experiment are summarized in figure 1. The data are plotted in mean values for each group of three cows, for the sake of simplicity. The curves are conclusive in showing the downward trends of the fat percentage during the feeding of the oil and the normal trends during consumption of the hydrogenated product. The mean fat percentages

for each cow during each week of the study are given in table 1, to illustrate the responses in each case. In every one of the six cows, the cod liver oil produced the usual effect in lowering the fat percentage of the milk and in elevating the iodine number. The hydrogenated cod liver oil had no effect upon the secretion of the milk fat and little if any on the iodine number of this fat. Furthermore, the animals

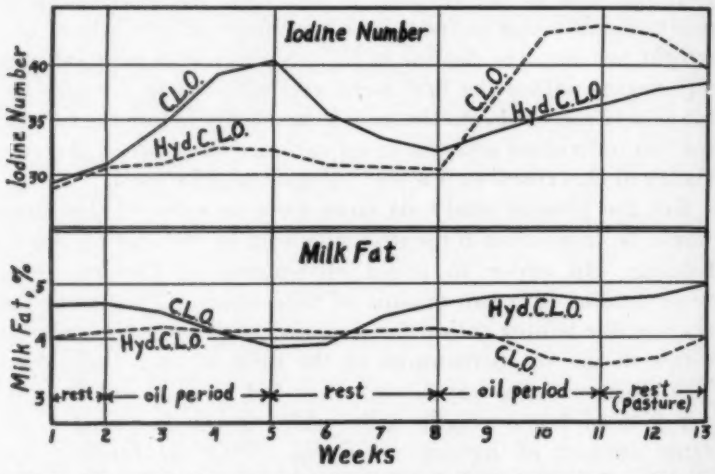

Fig. 1 The effect of feeding cod liver oil and the hydrogenated product to lactating cows. The ordinary decreases the secretion of milk fat while the hydrogenated does not. The former also increases the iodine number of the milk fat much more than the latter.

TABLE 1

Mean fat per cent in the milk of each cow during each week

COW	HERD RATION		COD LIVER OIL			HERD RATION			HYDROGENATED COD LIVER OIL			PASTURE	
G	4.60	4.58	4.59	3.98	3.96	3.98	4.49	4.51	4.93	4.84	4.66	4.67	4.83
F	5.60	5.70	5.60	5.57	4.87	5.03	5.54	5.74	5.92	5.86	5.50	5.78	6.62
C	3.70	3.75	3.38	2.90	2.68	2.73	3.24	3.62	3.90	3.70	3.82	4.01	3.87
			HYDROGENATED COD LIVER OIL						COD LIVER OIL				
T	4.30	4.69	4.69	4.50	4.83	4.71	4.55	4.58	4.63	4.21	4.20	4.19	4.46
L	3.89	4.04	3.97	3.87	3.85	3.76	3.82	4.13	3.75	3.43	3.25	3.70	3.86
E	3.93	3.87	4.12	3.82	3.95	3.76	4.08	3.90	3.80	3.35	3.07	3.07	3.54

regularly exhibited their distaste for the regular oil but no reaction toward the hydrogenated product.

THE PRODUCTON OF MUSCLE LESIONS

In an earlier paper the production of Agduhr's muscular degeneration by cod liver oil was described and the literature in this field reviewed (Madsen et al., '35). In the course of a number of years devoted to this problem, it has been found that guinea pigs fed synthetic diets are very sensitive test animals. They respond in a relatively short time. Their reaction to the feeding of cod liver oil is much less uniform than that of the lactating cow, however, probably because the feeding of synthetic diets to Herbivora has not been sufficiently developed.

To compare the toxicity of the two forms of cod liver oil previously fed to cows, ten guinea pigs were divided into two groups by age and sex. The following basal diet was used. This consists of casein 15, sucrose 15, starch 35, mineral mixture 4, regenerated cellulose 15, agar 5, irradiated yeast 7, and tomato juice (2 cc. per day per 100 gm. of body weight).

Group 1 was fed this basal diet mixed with 4% of the cod liver oil, while group 2 was fed this same basal with 5% of the hydrogenated product. The higher level of the latter was used to compensate for any likely increased excretion in the feces and to make the test more rigid. To the hydrogenated oil enough carotene was added to provide 100 units of vitamin A per day to compensate for probable loss in this factor during processing. For histological study, the gastrocnemius and vastus quadriceps muscles were fixed in Zenker's solution and treated in the usual manner. Experience has shown that these muscles reflect the action of the cod liver oil in such experiments.

The results of this study are summarized in table 2. The number of animals is small but the trend is clear. The tendency of the guinea pigs to die and to exhibit muscle lesions if they live over 2 weeks when fed cod liver oil is evident, in agreement with our previous findings. On the

other hand, it is questionable if any of the animals fed the hydrogenated product developed muscle lesions. Furthermore at the end of 2 months there were no animals alive in the cod liver oil group, while three were alive in the other. The body weight data which are not reproduced here indicated the growth and development of those fed hydrogenated cod liver oil and the failure of the other group. The beneficial

TABLE 2

Post mortem data on guinea pigs fed regular and hydrogenated cod liver oils

GROUP	ANIMAL NO.	DAYS OF EXPERIMENT	MANNER OF DEATH	LESIONS, VASTUS QUADRICEPS AND GASTROCNEMIUS MUSCLES [1]	NOTES
I	2	7	Died	No sections	Refused to eat
Regular	5	16	Died	+ (slight)	Hard dry feces—impaction
cod liver	3	18	Died	+ to ++ (areas)	
oil	4	31	Died	+++ to ++++	
	1	57	Killed	+++	Moribund—could not walk
II	7	21	Died	Negative [2]	Diarrhea
Hydro-	9	24	Died	Isolated degenerated fiber [2]	Impacted large intestine
genated cod liver oil	6	60	Killed	Negative	Slightly mottled liver
	8	60	Killed	Negative	Normal
	10	60	Killed	Negative	Normal

[1] The lesions are classified as + to ++++ according to degree of injury, as in our previous publication (Madsen et al., '35).

[2] These tissues had areas that were not strictly normal, but the very slight changes shown may have been due to difficulties in fixing.

effect of the hydrogenation seems clear. Much longer periods of observation would be needed to decide whether it entirely eliminates the toxic factor.

THE FAILURE OF YEAST TO COUNTERACT THE EFFECT OF COD LIVER OIL UPON MILK FAT AND THE FAILURE OF SALMON OIL IN SHOWING THE REACTIONS OF COD LIVER OIL

From the early studies of Agduhr ('28) as well as ourselves (Madsen, McCay and Maynard, '35), it is evident that the composition of the basal diet as well as the cod liver oil is responsible for the production of degenerated muscles. This

has been amply confirmed in later work both in our laboratory and by Madsen ('36). The literature furnishes some indication that yeast can partly counteract the toxic action of cod liver oil in the development of muscle lesions, but furnishes no data concerning any interaction between cod liver oil and other factors in the diet of the cow. For this reason experiments were run with a group of cows to determine if there were any indications that when yeast and cod liver oil were both fed the secretion of milk fat might fail to decrease in the usual manner.

In this experiment six cows were divided into two groups of three each. One group was fed cod liver oil at a level of ½ cc. per kilo of live weight and as much yeast [3] as each individual could be induced to eat. This yeast was first mixed with the grain mixture and later with the silage. By this means each cow was fed from ½ to 1 pound daily.

Since cod liver oil alone is very consistent in reducing the milk fat, it was considered more profitable in this preliminary trial to use another fish oil as a control. Thus the other group of three cows was fed salmon oil at the same level as the cod liver oil, but received no yeast. This experimental period lasted for 3 weeks. From these preliminary experiments no evidence was obtained that yeast had any effect in counteracting the action of cod liver oil in lowering the secretion of milk fat. The fat percentage dropped in the usual manner and the iodine number of this fat increased. During the 3 weeks following the feeding of the oil and yeast the milk fat secretion returned to its earlier level, as if cod liver oil had been fed alone. No further studies were made with yeast since these preliminary ones had given no evidence of activity in counteracting the effect of cod liver oil.

The results with the salmon oil were conflicting. The milk fat in one case dropped suddenly the first week and then arose, in the course of the next two weeks, to the initial level. In a second cow the fat remained at a constant level for the first

[3] The dried yeast was obtained through the courtesy of C. N. Frey of the Fleischmann Company.

2 weeks of the oil feeding and then dropped in a manner comparable to that caused by cod liver oil. In the case of the third cow the curve for the drop of milk fat resembled that which is obtained in almost every case of feeding cod liver oil to a lactating cow.

These results indicated, like our earlier studies with this product, that it either lacked the factor of cod liver oil or had it present at a much lower level. Therefore another experiment with salmon oil was run. In this case four cows were fed salmon oil as a supplement to their usual feed at a daily level of $\frac{1}{3}$ cc. per kilo of body weight. After a preliminary period, the oil was fed daily for 3 weeks. This in turn was followed by a rest period with no oil. The results were conflicting again. One cow was unaffected and the fat level of the milk remained unchanged. Two exhibited a slight lowering at the end of 3 weeks and for the fourth the curve resembled that for cod liver oil. All samples of milk fat tended to exhibit an increase in the value of the iodine number during the salmon oil feeding and this value dropped back to the normal for the individual and ration in the course of the first week after the oil feeding stopped. No explanation for these conflicting results in milk fat secretion could be found in such factors as disturbance of the cows or volume of milk secreted.

No further studies were made with salmon oil but it is evident that higher levels than $\frac{1}{3}$ cc. per kilo must be employed. Since salmon oil is from the body as well as from the liver, it is possible that the fish liver contributes the active factor rather than the fish body as a whole.

SUMMARY

Two experiments were run with lactating cows to determine the effect of feeding hydrogenated cod liver oil upon the secretion of milk fat. In contrast to the original oil, the hydrogenated product neither lowered the fat percentage of the milk nor significantly raised the iodine number of the milk fat. Limited evidence from feeding this hydrogenated

oil to guinea pigs also indicated that it does not produce muscle lesions over a period of 60 days, although such lesions are regularly produced with the oil before hydrogenation. The feeding of from ½ to 1 pound of dried yeast daily with the usual amount of cod liver oil did not counteract the lowering of the milk fat caused by the oil in lactating cows. Two additional studies of the influence of salmon oil on milk fat secretion in cows indicated that this oil does not react like cod liver oil. If the injurious factor is present it is probably in a lower concentration.

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THE EFFECT OF MELTING POINT OF FAT UPON ITS UTILIZATION BY GUINEA PIGS

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(Received for publication October 18, 1937)

For 50 years or more it has been recognized that one of the factors which determines the utilization of fat by an animal body is the melting point of this fat. Arnschrink (1889) was one of the early workers who classified fats into three groups upon the basis of their melting points and relative utilization within the body. Most of these early studies were made with dogs. Recent studies of Steenbock, Irwin and Weber ('36) indicate that the rate of digestion and absorption of different fats within the body of the rat differs during the first few hours, but after 12 hours the differences are questionable.

A few balance studies have been made with men to determine the relative utilization of fats of different properties. For the most part, however, the periods used in such studies have been very short. Thus in the recent work of Massatsch and Steudel ('35), 3-day periods were employed in comparing the utilization of lard and hydrogenated fat.

Few studies of fat utilization by species other than omnivora or carnivora have been made. In studying the passage of fat from the feed into the milk of goats Gogitdse ('04) concluded that higher melting fats were absorbed with difficulty in the case of goats.

In experiments with brook trout to determine the relative utilization of hard and soft fats, McCay and Tunison ('34) found that oils such as cottonseed or salmon oils were always better utilized than hard fats such as hydrogenated ones. In

the case of brook trout this might be anticipated since the processes of the body operate at a temperature of 8°C. These reactions in the body of the fish have been reviewed recently (McCay, '37).

To study this matter further exploratory experiments were made with rats and guinea pigs. Four adult rats were fed dry skimmed milk for a period of 10 days to establish the intake. In a following period of 10 days approximately 20% of the calories was replaced by cottonseed oil.[1] The last 5 days of this period the feces were collected. During the following

TABLE 1

Utilization of the fat in diets of skim milk milk and cottonseed oil and skim milk and hydrogenated oil by rats

	RAT NO.	FAT INTAKE MILK	(5 DAYS) FAT	FAT IN FECES (5 DAYS)	UTILIZATION
		gm.	gm.	gm.	%
Cottonseed oil	1	0.261	3.310	0.076	97.9
	2	0.194	3.128	0.113	96.6
	3	0.286	3.300	0.123	96.6
	4	0.267	3.152	0.052	98.5
Hydrogenated oil	1	0.260	3.011	0.165	95.0
	2	0.252	3.020	0.144	95.6
	3	0.308	3.470	0.134	96.5
	4	0.187	3.027	0.248	92.3

10 days hydrogenated fat[2] was fed similarly and the feces collected during the latter half of the period. The data from this experiment are summarized in table 1. These results indicate that the rat is able to use both fats quite effectively.

In the case of guinea pigs[3] another technic was employed. A diet low in fat was first prepared by extracting a mixture of grain and ground alfalfa with isopropyl ether. This extracted mixture was then supplemented by the feeding of 10 cc. of tomato juice daily to each animal. Care was used to

[1] Wesson oil was the product used.

[2] Crisco was the product used.

[3] These preliminary studies with guinea pigs were made by Mrs. L. Dudgeon and Miss M. Zoller.

moisten this grain mixture to insure adequate consumption. The experiment was divided into three periods of 11 days each. During the first, the low fat diet was fed. The feces were collected during the last 6 days of the period. During the next period the feed was mixed so as to contain 6% of cottonseed oil and the feces collected again during the last 6 days. In the final period the feed contained hydrogenated oil[4] at the same level instead of the cottonseed oil. Three guinea pigs were used in these experiments.

The feces were preserved in alcohol then dried in vacuo and ground. Samples were then extracted with ether in the

TABLE 2

Relative utilization by guinea pigs of oil before and after hydrogenation

Diet	*Lipids in dry feces, per cent*
Basal (extd.)	0.96
	0.97
	0.80
Basal plus cottonseed	1.69
	1.85
	1.85
Basal plus hydrogenated oil	5.43
	5.86
	5.46

usual manner. Table 2 shows the variations in the fat content of the feces, although the weight of dried feces excreted was but little different in the various periods.

Further calculations indicated that in this trial about 90% of the oil was utilized and only about 60% of the hydrogenated product. These values indicate the differences clearly but are probably much less accurate than later ones that were obtained after a couple of years additional experience in feeding guinea pigs these special diets.

In order to explore this field further, a new series of preliminary trials with a number of different oils was run using essentially the same technics. Details will not be reported since the later experiments were similar and somewhat more

[4] The hydrogenated oil used was Crisco.

accurate. In this second preliminary series were included not only cottonseed oil and its hydrogenated product but also shark liver oil, soybean oil, castor oil, palm kernel oil, hydrogenated cod liver oil, cod liver oil, olive oil, butter, cocoanut oil and peanut oil. These preliminary trials yielded some surprising results in addition to confirming the first findings, namely, that hydrogenated oils are not utilized to the same degree as lower melting ones. Castor oil not only seemed to have no cathartic action upon the guinea pigs but apparently was utilized as well as such oils as shark liver and peanut.

TABLE 3

Utilization by guinea pigs of various fats as supplements to a low-fat diet

Fat supplement in diet	*Fat in feces, per cent*	*Utilization, per cent*
Castor oil	1.25	96.2
Soybean oil	1.54	94.5
Olive oil	1.60	94.5
Cocoanut oil	1.69	94.0
Salmon oil	2.03	94.0
Cod liver oil	1.65	93.8
Neatsfoot oil	1.64	93.5
Peanut oil	2.33	91.8
Butter	2.58	91.0
Cottonseed oil	3.36	87.4
Corn oil	3.35	86.5
Mutton tallow	5.22	79.8
Lard	6.70	75.2
Hydrogenated cottonseed oil	8.42	73.8
Beef tallow	7.05	72.0

A third experiment was now run taking advantage of earlier experience in feeding the animals. Ten kilograms of the basal diet were first extracted and dried carefully to free it from isopropyl ether. Three healthy guinea pigs were selected that would consume the diets readily. Each lipid to be tested was incorporated to comprise 6% of the diet. This mixture was then fed for a period of 11 days. The feed intake was determined and the feces collected during the last 7 days of each period. Since these animals were maintained upon the diets for a long period of time, they were fed adequate supplements of an A-D concentrate and tomato juice, allowing 8 cc. of the latter per day.

The data from this series are summarized in table 3. The results are very similar to those of the two preceding experiments. The guinea pig seems unable to utilize higher melting fats as effectively as oils. In this series again the castor oil seemed to be well digested and absorbed. No evidence of a cathartic action was found.

No corrections were made for metabolic fat in these studies since the results are evident without such corrections and since calculations involving metabolic fat make the assumption that the excretion of fat into the intestine is the same during periods of low fat and of normal fat ingestion.

TABLE 4

Fecal lipids as determined by the Tidwell-Holt method and by the regular ether extraction procedure

Oil supplement	*Tidwell-Holt method, per cent*	*Ether extract method, per cent*
Castor oil	2.31	1.25
Olive oil	2.34	1.60
Cottonseed oil	3.75	3.36
Hydrogenated oil	9.15	8.42

In order to compare the amount of lipid extracted from the feces by ethyl ether and another procedure, a few typical fecal samples were run by the method of Tidwell and Holt ('36). The values of table 4 show the latter method yields somewhat higher values but the differences between the oils and higher melting fats show the same trend for both methods.

SUMMARY

In order to determine if the melting points of a fat in region of body temperature is important in influencing its utilization in Herbivora, a series of balance studies was made chiefly with guinea pigs. The following oils were fed, incorporated at a 6% level in a diet of alfalfa hay and grain which had been extracted previously with isopropyl ether: castor, soybean, olive, cocoanut, salmon, cod liver, neatsfoot, peanut, butter, cottonseed, hydrogenated cottonseed, corn, tallow and lard. The fecal lipid tended to be much higher after feeding

the higher melting fats. The higher melting fats are not so well utilized as the oils by the guinea pig in contrast to the rat. Even castor oil seems well absorbed.

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THE RELATION OF CELLULOSE AND LIGNIN CONTENT TO THE NUTRITIVE VALUE OF ANIMAL FEEDS[1]

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For the past 40 years, animal feeding stuffs have, for general description, been divided by proximate analysis into six fractions: moisture, ether extract, protein, ash, crude fiber and nitrogen-free extract. This analysis has been the common basis of the nutritional classification of feeds, particularly the three fractions (protein, fat and fiber) which have been used in feed control measures. Through an extension of this analysis to the feces voided by animals on controlled feeding tests, the usual digestibility coefficients for feeds are calculated. The significance of the specific values obtained, however, has frequently been over-estimated. This has been true especially of the crude fiber fraction.

In terms of feeding value, crude fiber is intended as a measure of the quantity of the fibrous, poorly digested material in the feed. On the other hand, one has but to recall the chemical procedure by which this fraction is isolated to understand that any relation crude fiber may have to the digestibility of a feed may be in part fortuitous. (For a full discussion of this subject, the reader is referred to the work of Norman, '35).

[1] The experimental work of this study was carried out largely in the Laboratory of Animal Nutrition, Cornell University, and excepting where otherwise noted, the data are taken from a thesis presented by E. W. Crampton in partial fulfillment of the requirements for the degree of doctor of philosophy.

That the digestibility of the dietary carbohydrate does not follow its partition into crude fiber and nitrogen-free extract with any marked certainty, especially in the case of roughages, is evident from table 1 calculated from digestion coefficients reported by Morrison ('37).

The published literature also contains ample evidence that the crude fiber of forages may be as well digested as the protein (Newlander and Jones, '32; Mitchell and Hamilton, '33; Morrison, '37). It would, therefore, seem that the justification for partition of the carbohydrate fraction of feeds into crude fiber as the poorly, and nitrogen-free extract as the highly digestible parts, may be open to some question.

TABLE 1

Relative digestibility of Weende crude fiber and nitrogen-free extract

KIND OF FEED	NUMBER AVERAGED	AVERAGE COEFFICIENT OF DIGESTIBILITY		PER CENT OF CASES WITH CRUDE FIBER SHOWING AS COMPLETE DIGESTIBILITY AS N-FREE EXTRACT
		Crude fiber	N-free extract	
		%	%	%
Dry roughages	110	52.4	59.5	39
Green roughages	61	63.5	76.3	20
Silages	25	58.2	64.6	28
Concentrates	88	53.3	78.5	10
All feeds	284	55.6	69.5	25

Furthermore, it is logical to believe that if a division of the carbohydrate fraction could be made into parts which were either biological or chemical units, the usefulness of the feeding stuffs analysis in predicting probable feeding value would be enhanced. The problem of such a partition resolves itself largely into a consideration of the chief constituents of the 'cell wall' carbohydrates—cellulose, hemicellulose and lignin.

Chemical and biological nature of lignin, cellulose and hemicellulose

Lignin occurs in plants chiefly as lignocellulose. There is support for the belief that substances of the glucosanxylan series are the forerunners of lignin, but neither its exact

chemical structure nor the manner in which it is combined with cellulose are fully understood. Its behavior in nutrition is likewise unsettled, different feeding tests yielding conflicting results (Dietrich and König, 1871; König and Becker, '18; Paloheimo, '25; Rogozinski and Starzewska, '27; Rubner, '28; Phillips, '29, '34; Fuchs, '36; Woodman and Stewart, '32; Prjanischnikow and Tomme, '36). Proof for or against its utilization by the animal is difficult to establish, for until its molecular structure is known, no criterion of the accuracy of a quantitative test for lignin is possible.

In preliminary tests of this study, and using chemical procedures to be later described, 97.8 and 99.3% of the dietary lignin were recovered in the feces of rabbits and a steer, respectively. Subsequent tests of the digestibility of certain dietary constituents of mixed pasture herbage clippings substantiated this finding with rabbits as seen in the following figures:

Date of clipping of grass fed	*Per cent dietary lignin recovered in feces* [1]
May 12	97.56
June 3	95.18
June 20	93.39
July 9	100.32
July 9	103.67
July 24	96.87
August 20	102.73
Average	98.53

These data support the rather more general opinion that dietary lignin is not appreciably metabolized by animals.

Cellulose. Neither the chemical nature of cellulose, nor the steps by which it eventually yields products nutritionally useful to an animal, can be discussed here (see Pochon, '35; Woodman and Stewart, '28). Undoubtedly the extent to which such end products are produced may be influenced by a number of factors (Mangold, '34). Among them, the possible role

[1] Digestibility data from nutrition laboratory, Macdonald College and obtained for each clipping by pooling feed and feces figures for three rabbits fed individually on identical diets. The rabbit feeding was carried out by Mr. Robert P. Forshaw and the analyses of the feces by Mr. A. J. Sutherland, graduate assistants at Macdonald College. Their assistance is much appreciated and gladly acknowledged.

of lignin is of special interest in this study. Lignified plant tissues apparently are not attacked by alimentary bacteria (Woodman and Stewart, '32), perhaps because of a certain degree of antiseptic action of the lignin resulting from its phenolic nucleus (Hibbert).[3]

This obviously suggests that if lignification differs in degree between samples of forage either because of species or environmental conditions, the feeding value of such material may be changed as a result. This might, in part, explain the observed decrease in nutritive value of pasture grasses during seasons or parts of seasons when conditions are not favorable to rapid growth, but instead favor lignification of the plant tissues. On the other hand, it may not follow that, other factors constant, a decrease in digestibility of the feed will reflect an increase in total lignin, inasmuch as the manner of its deposition in the plant may be a factor of importance in this respect (Woodman and Stewart, '32).

Hemicellulose, according to Armstrong and Armstrong ('34), is perhaps most usefully visualized as consisting of a mixture of the components of incompletely formed woody substances containing, in association, true cellulose chains, xylan and polyglucuronates (see also Norman, '36). The usually accepted method of quantitative estimation (furfuraldehyde) is open to the criticism that hemicellulose, of animal forage at least, contains appreciable quantities of hexosans as well as pentosans. Biologically, this group of carbohydrates follows much the same paths of degradation as cellulose. However, there is some evidence (Schmidt-Ott, '36; Iwata, '35; Williams and Olmsted, '35) that they may be somewhat more completely utilized by the animal system, thus standing intermediate between cellulose and the easily soluble starches and sugars.

The large number of substances which appear logically to be classed as hemicellulose, together with the confusion which at present exists in the exact definition of this group, makes

[3] Hibbert, H. Pulp and Paper Research Institute, McGill University, personal communication.

the possibility of a satisfactory chemical estimation rather remote. There appears to be no advantage for purposes of a feeding stuffs analysis in determining a part of the group, as, for example, pentosans, and still leaving an undetermined fraction. As an alternate plan, estimation by difference of the total hemicelluloses plus any other carbohydrates not cellulose, seems to be a more logical procedure and was adopted in this study.

Chemical estimation of lignin and cellulose

Chemical determination of lignin. Space does not permit a full discussion of the problems of lignin determination, and for such material the reader is referred especially to papers by Norman and Jenkins ('34), Goss and Phillips ('36), Cohen ('36), Cohen and Harris ('37), Waksman and Stevens ('30), Ross and Potter ('30), and Ross and Hill ('29). Consideration of these and other studies led us to the conclusion that at the present time some form of the '72% H_2SO_4' method is the most satisfactory for the quantitative estimation of the lignin content of animal forage. It consists in solution in concentrated H_2SO_4 of the sample which has been pretreated to remove fats, sugars and proteins which otherwise would interfere with the lignin values obtained.

The special problem, in the case of forage and animal feces, lies in the removal of the protein without a simultaneous removal of a part of the lignin. According to present information, lignin is soluble in varying degrees in dilute alkali (hot or cold), boiling water, and dilute mineral acid (1.25% H_2SO_4) at boiling temperature. Pretreatment by enzyme digestion, however, would seem to be a suitable possibility. Williams and Olmsted ('35), working with human diets and feces, proposed the use of pancreatin in a solution buffered at pH 8. In their proposal, cellulose and hemicellulose were determined on the filtrate remaining on removal of the lignin; but because some hemicellulose is removed by the alkaline pancreatin solution, a correction is made by running an additional sample of the diet with the pancreatin omitted.

Lignin values are taken from the sample digested with the pancreatin. But if lignin were isolated from both the enzyme and no-enzyme residues, any effect of the pretreatment on lignin would be seen. (Fats, starches and soluble sugars are removed by ether extraction and autoclaving previous to the enzyme treatment.)

When this procedure was tried with sheep and steer diets of grain and hay, the results in every case showed a smaller lignin value in the pancreatin treated sample, as indicated in table 2.

TABLE 2

Effect on lignin values of pretreatment of sheep and steer diets by pancreatin digestion

DESCRIPTION	LIGNIN (AS PER CENT OF AIR DRY SAMPLE)	
	With pancreatin	Without pancreatin
	%	%
Sheep diet—grain + alfalfa hay	4.76	5.40
Sheep diet—grain + timothy hay	4.21	5.78
Steer diet—grain + alfalfa hay	2.81	3.40

It seems probable that the long exposure of the samples (72 hours at 45°C.) in a solution buffered at pH 8 using NaOH as the alkali, may have dissolved some of the lignin and thus resulted in the lowered lignin values.

Another difficulty was also encountered in the use of the Williams and Olmsted procedure. It was found impossible in the case of the animal diets to effect complete solution in the concentrated H_2SO_4 of the enzyme digest residue. Subsequently, when a lignin balance was struck from a steer digestion trial, 25% more lignin was recovered in the feces than was consumed.

Pepsin, on the other hand, is active in acid medium; and it has not yet been shown that lignin is soluble in dilute mineral acid at temperatures at which this enzyme is active. The effectiveness of this enzyme in removing proteins was uncertain in view of studies by Horwitt et al. ('36) in which not more than 89% of the nitrogen was removable from spinach leaf by pepsin digestion. There was the possibility, however,

that the protein of the materials would be reduced by pepsin digestion to a level no longer seriously interfering with lignin determination. If the hypothesis is accepted that lignin is not utilized by the animal, the usefulness of the pepsin pretreatment and subsequent analytical steps would be indicated by a lignin balance trial. Such a test yielded the data given in table 3. The practically quantitative recovery of the dietary lignin obtained argues well for the procedure used.

TABLE 3

Digestibility of lignin by rabbits and a steer

ITEM		RABBITS	STEER
Dry matter	Consumed	228 grams	53.00 pounds
	In feces	79 grams	10.75 pounds
Per cent lignin	In feed	9.03%	5.56%
	In feces	25.48%	27.12%
Weight of lignin	Consumed	20.58 grams	2.95 pounds
	Voided	20.15 grams	2.92 pounds
Balance		0.43 grams	0.03 pounds
Per cent recovery		97.8%	99.3%

The problem of completely and rapidly dissolving the undigested residue in the strong acid was solved by Ross and Hill ('29) who found that lignified tissues would dissolve promptly (10 to 15 minutes) in 72% H_2SO_4 if first moistened with formalin. With the sample in contact with the acid for so short a time, the difficulty with the formation of substances from the carbohydrates (pentoses or hexoses) which might add to the lignin value is presumably largely avoided (Ritter et al., '32). The use of a granulating reagent (chloroform-acetic acid) in the precipitation of the lignin to hasten the time necessary for filtration was proposed by Ross and Potter ('30), and has been recommended by Baillie ('36), in a microtechnic. It appeared, therefore, that pepsin digestion of the ether extracted sample, followed by solution of the residue in 72% H_2SO_4 and subsequent precipitation of the lignin according to the Ross and Hill, and Ross and Potter procedures could be successfully used for lignin determination in animal feeds and feces.

Specifically, the procedure eventually adopted in this study is as follows: Place the oven-dry, ether extracted residue from a 1 gm. sample of feed (or feces) in a 50 cc. glass stoppered Erlenmeyer and add 40 cc. of a 2.0% solution of pepsin in N/10 HCl. Digest for 12 hours at 40°C., shaking frequently especially during the first 4 or 5 hours. Recover the non-digested residue by filtration through bolting silk and wash successively with hot water, hot alcohol, hot benzene, hot alcohol, and ether. Transfer the washed residue to a 100 cc. beaker, and remove the last traces of ether with mild heat. Moisten the residue with 4 cc. of 40% formaldehyde. Then add 4 cc. of 72% H_2SO_4, and allow it to penetrate the sample (2 minutes). Add 6 cc. of concentrated H_2SO_4 and stir vigorously with a glass rod to aid in solution of the sample which should be complete in 10 to 15 minutes. Partially immerse the beaker in a cold water bath, if necessary, to prevent the temperature from rising above about 70°C. When dissolved, stir in 35 cc. of a granulating reagent consisting of a 1:6 mixture (by volume) of chloroform and acetic acid, and then pour the whole into 500 cc. distilled water in an 800 cc. beaker. Boil gently until the chloroform has been driven off (15 minutes), after which the solution should clear and the lignin settle in granular form. Filter on a Gooch with suction. Wash in not less than 200 cc. of 5% HCl. Dry at 110°C. and determine lignin by loss on ignition. This procedure has given reproducible results in the hands of two analysts.

Determination of cellulose. For a summary of the many methods which have been used for the quantitative estimation of cellulose, the reader is referred to papers by Kürschner and Hoffer ('31), and Kürschner and Hanak ('30). According to the former procedure, the sample was freed of non-cellulose, organic constituents by digestion with an alcohol-nitric acid reagent. The treatment involved boiling the sample with the reagent for two and sometimes three successive hour periods. In the latter procedure, acetic acid is substituted for the alcohol in the digestion reagent and the time of treatment reduced to 20 minutes.

Comparative tests of these two procedures resulted in practically the same values for cellulose from feces material, but lower values from certain feeds with the acetic acid-HNO_3 reagent, as is shown in table 4.

TABLE 4

Cellulose as per cent of moisture-free sample

	ALCOHOL-HNO_3	ACETIC ACID-HNO_3
	%	%
Steer feces	21.7	21.1
Rabbit feces	30.4	31.0
Steer feed (grain + alfalfa hay)	14.7	11.4
Rabbit feed (grass clippings)	22.4	22.3

A possible explanation of this might lie in a difference in the resistance of the cellulosan fractions of mature hay and grain as compared to those in immature grasses. Certainly, the CH_3COOH-HNO_3 mixture is the more powerful reagent. Incidentally, the results with the feces might be interpreted that the animal was able to remove most of the dietary cellulosans.

These results, together with its greater simplicity, led to the choice of the acetic acid reagent for the cellulose determination. It was found, however, that by using alcohol instead of water for the first washings to free the cellulose from the digesting reagent, this process could be carried out by centrifuging, which considerably facilitated the washing operations.

The details of the procedure used are as follows: Place a 1 gm., air dry sample in a 150 cc. round-bottomed, wide-necked flask fitted with a reflux condenser. Add 15 cc. of 80% acetic acid and 1.5 con. HNO_3. Boil gently for 20 minutes. Transfer the sample and liquid to a 50 cc. centrifuge tube; add about 20 cc. of alcohol and centrifuge 10 minutes. Decant the liquid. Wash (in centrifuge tube) with alcohol. Transfer the residue (with aid of a stream of alcohol from a wash bottle) into an alundum crucible and wash successively with hot benzene, hot alcohol, and ether—using suction. Dry. Calculate cellulose

as loss on ignition. Our experience has shown this method to give reproducible results.

A test of a modified procedure for feeding stuffs analysis

This study was originally undertaken in connection with the problem of the nutritive value of pasture herbage where the

TABLE 5

Analysis of feeds and feces and apparent digestibility of diets

ANIMAL	PROCEDURE		ANALYSIS		PER CENT DIGESTIBILITY
			Per cent diet	Per cent feces	
Yearling Angus steer weight 850 pounds	Standard	Ether extract	6.59	7.61	76.6
		Protein (N×6.25)	12.19	18.73	68.8
		Ash	3.64	9.91	...
		Crude fiber	12.11	25.66	57.0
		N-free extract	65.47	38.08	88.2
Diet: equal parts alfalfa hay and grain	Modified	Ether extract	6.59	7.61	76.6
		Protein	12.19	18.73	68.8
		Ash	3.64	9.91	...
Dry matter eaten: 53 pounds		Lignin	5.56	27.21	0.7
		Cellulose	11.42	21.12	62.5
Dry feces: 10.75 pounds		Other carbohydrate	60.60	15.42	94.8
Mature rabbit	Standard	Ether extract	5.10	5.60	61.9
		Protein	23.75	13.15	80.8
		Ash	9.80	13.60	...
		Crude fiber	20.10	28.00	51.7
		N-free extract	41.25	39.65	66.7
Diet: pasture grass clippings	Modified	Ether extract	5.10	5.60	61.9
		Protein	23.75	13.15	80.0
Dry matter eaten: 228 gm.		Ash	9.80	13.60	...
		Lignin	9.03	25.48	2.2
Dry feces 79 gm.		Cellulose	22.35	30.40	52.9
		Other carbohydrate	29.97	11.77	86.4

lack of correlation between the 'standard' feeding stuffs analysis and feeding value as measured by the growth of rabbits had been especially noted. To test the value of the data obtained by modifying the standard method of analysis by the inclusion of the determinations for cellulose and lignin, analyses were made of the feed and feces from a digestion trial with rabbits and a steer. The feeds and feces were

analyzed for water, protein, ash, fat and fiber by the usual procedures and for cellulose and lignin by the methods previously outlined. The term 'other carbohydrates' was used to denote the residue obtained by difference. These data are presented in table 5.

These data suggest that the modified procedure makes a sharper distinction among the carbohydrates as regards their digestibility. The carbohydrates are partitioned into three fractions: a practically non-digested portion, lignin; a highly digestible fraction, other carbohydrates; and into cellulose which is both biologically and chemically a recognizable unit. The digestibility of the latter fraction by a given animal may be expected to vary inversely with the degree (or nature) of its lignification.

As a means of estimating their feeding value, the modified scheme of analysis here proposed would seem to offer distinct advantages over the standard procedure for the analysis of rations, especially of Herbivora. Further study is needed of the application of the methods proposed to various kinds of feeds, and of the correlation of the analytical data with the results of feeding trials. Such studies are now in progress at Macdonald College.

SUMMARY

Data are presented which show the limitations, as measures of digestibility and nutritive value, of the crude fiber and nitrogen-free extract partition of the carbohydrates in animal feeds. Based on studies of various procedures, methods are proposed for the determination of cellulose and lignin in feeds and feces. The usefulness of the inclusion of these determinations in feed analyses is shown by data obtained in digestion trials with rabbits and steers. The data indicate that, at least for Herbivora, a partition of the carbohydrate portion of a feed into lignin, cellulose and other carbohydrates may have more biological significance, and hence be of greater usefulness in predicting feeding values than the present division into crude fiber and nitrogen-free extract.

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EFFECT OF FEEDING HIGH LEVELS OF COPPER TO ALBINO RATS[1]

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(Received for publication October 18, 1937)

It is now a generally accepted fact that copper is an essential element and that it functions specifically in the stimulation of hemoglobin formation in the presence of an otherwise adequate diet (Harrow and Sherwin, '35). Schultze, Elvehjem and Hart ('34) have called attention to the fact that in the rat, quantities of copper as low as 0.005 mg. per rat per day will give a distinct response, while the optimum level is between 0.01 and 0.05 mg. per day. Since it is commonly believed that copper is toxic, it seems desirable to ascertain the level at which this essential element begins to exhibit toxic effects.

Waddell, Steenbock and Hart ('31) observed that rats could tolerate much more copper when their daily dosage was mixed with a dry ration than when it was fed all in 1 dose in a small amount of milk. When the latter technic was used, levels of 4 and 16 mg. of Cu per day were very toxic for rats. Earlier work by Lindow, Peterson and Steenbock ('29) indicated that 5 mg. of Cu per day was tolerated by rats when the copper was mixed with a dry diet. Coulson, Remington and Lynch ('34) observed no toxic effects in rats fed a diet containing 527 parts per million of copper as $CuSO_4$.

In the present work, copper sulfate was added to diets in amounts sufficient to give a copper intake of as high as

[1] Published with the permission of the director of the Wisconsin Agricultural Experiment Station.

[2] On leave of absence from the Kentucky Agricultural Experiment Station, Lexington, Kentucky, and published with the permission of the director.

40 mg. per day if food intake remained normal. Rats were fed ad libitum diets in which the copper was uniformly mixed with the ration. Thus the animals established their copper intakes voluntarily and the resulting intake was determined by accurate food consumption records. In addition to growth records, the animals were sacrificed and the blood, liver and spleen were analysed for copper.

EXPERIMENTAL

White rats 21 days of age were placed on control ration no. 351 (Kline, Elvehjem, Keenan and Hart, '34) minus cod liver oil. Throughout the experimental period vitamins A and D were administered orally in the form of percomorph oil. After 1 week the rats were divided into groups and placed on diets containing 0, 500, 1000, 2000 and 4000 parts per million of copper as copper sulfate. The diet containing the highest level of copper was prepared by pipetting an exact amount of copper sulfate solution on the diet, which was then dried and pulverized. The other diets were prepared by mixing various proportions of the most toxic diet with the control diet.

TABLE 1

Average growth, food intake and copper intake of rats receiving high-copper diets

SERIES NO.	NUMBER ♂	NUMBER ♀	COPPER ADDED TO BASAL RATION	AVERAGE GROWTH IN 4 WEEKS	AVERAGE FOOD INTAKE	AVERAGE EXCESS Cu INTAKE
			mg. per kg.	*gm.*	*gm./rat/day*	*mg./rat/day*
8900	1	2	0	86	11.8	0
9000	4	1	0	126	11.8	0
8900	1	2	500	78	10.2	5.1
9000	3	2	500	89	10.1	5.05
8900	1	2	1000	51	8.2	8.2
8900	1	2	2000	5.0	4.9	9.8
9000	4	1	2000	6.0[1]	5.9	11.8
8900	1	2	4000	—6.7[2]	1.9	7.6

[1] One died in fourth week.
[2] All died in first week.

Two series were run. In the first (S8900) the rats on 4000 ppm. of copper died in 1 week and this level was there-

TABLE 2

Copper content of blood, spleen and liver from rats receiving high-copper diets

	BLOOD, MILLIGRAMS Cu PER 100 GM. OF BLOOD	SPLEEN			LIVER		
		Dry weight, grams	Milligrams Cu per		Dry weight	Milligrams Cu per	
			Whole spleen	100 gm. dried spleen		Whole liver	100 gm. dried liver
Control							
8942 ♀	0.128	0.333	0.00462	1.39	1.60	0.0217	1.36
8974 ♀	0.103	0.226	0.00294	1.30	1.80	0.0305	1.69
8964 ♂	0.0887	0.168	0.00291	1.73	2.23	0.0287	1.29
9051 ♂	0.0762	0.227	0.00235	1.04	2.37	0.0385	1.62
9052 ♂	0.104	0.188	0.00246	1.31	2.40	0.0397	1.65
9061 ♀	0.105	0.157	0.000747	0.477	1.69	0.0309	1.83
9072 ♂	0.0882	0.254	0.00258	1.01	2.66	0.0343	1.29
9064 ♂	0.102	0.239	0.000813	0.341	2.48	0.0291	1.17
Average	0.0994	0.224	0.00243	1.07	2.15	0.0317	1.49
500 mg. Cu per kilogram ration							
8944 ♀	0.146	0.160	0.00368	2.30	1.82	0.287	15.8
8973 ♀	0.183	0.107	[1]	[1]	1.38	0.278	20.1
8961 ♂	0.146	0.222	0.00266	1.20	2.45	0.761	41.1
9053 ♂	0.105	0.323	0.00278	0.860	2.54	0.410	16.1
9054 ♂	0.124	0.251	0.00167	0.664	2.11	0.654	31.1
9063 ♀	0.146	0.157	0.00214	1.37	1.78	0.223	12.5
9074 ♀	[1]	0.110	[1]	[1]	1.17	0.057	4.93
9071 ♂	0.123	0.205	0.00246	1.20	2.12	0.595	28.1
Average	0.139	0.192	0.00257	1.27	1.92	0.408	21.2
1000 mg. Cu per kilogram ration							
8945 ♀	0.228	0.107	0.00345	3.24	1.52	2.27	150
8972 ♀	0.188	0.103	0.00270	2.63	1.50	2.47	165
8952 ♂	0.247	0.159	0.00373	2.35	1.34	0.769	57.3
Average	0.221	0.123	0.00329	2.74	1.45	1.84	124.0
2000 mg. Cu per kilogram ration							
8946 ♀		0.0810	0.00284	3.51	0.914	4.10	448
8962 ♀	0.239	0.0403	0.00611	15.2	1.14	4.46	431
8955 ♂	0.431	0.0674	0.00216	3.20	0.801	3.29	411
9055 ♂	0.210	0.0715	0.00125	1.75	1.03	4.39	427
9056 ♂	0.190	0.121	0.00269	2.23	0.928	4.55	490
9062 ♀	0.123	0.0846	0.00246	2.91	0.684	2.91	425
9073 ♂	0.217	0.0845	0.00611	7.23	0.915	3.85	420
Average	0.235	0.0786	0.00337	5.15[2]	0.916	3.94	436

[1] Too low to read.

[2] If result on rat no. 8962 is not included the average is 3.46.

fore not used in the second series (S9000). The food consumption was determined by difference and extreme precautions were taken to eliminate spillage. The animals were weighed once a week. At the end of the fourth week the animals were anaesthetized with ether and blood was withdrawn for analysis using the method of Swanson and Smith ('32) to avoid any copper contamination and to obtain maximum amounts of blood. The rats were examined for gross pathology after which the livers and spleens were also removed for analysis. The blood and organs were analyzed for copper by the method of Fischer and Leopoldi ('34) using wet combustion with sulfuric and perchloric acids. The excess perchloric acid was removed with sodium sulfite. Whole spleens were analysed with 5 micrograms of copper added. Blood was analyzed in quantities ranging from 2 to 5 cc. Liver was analyzed using aliquots containing from 4 to 8 micrograms of copper. The growth, food intake, and copper intake of the various groups are shown in table 1. The results of the analyses are shown in table 2.

RESULTS AND DISCUSSION

It will be seen that the rats receiving 500 ppm. of copper injested about 5 mg. of copper daily and showed good growth and food consumption although both were slightly subnormal. The animals appeared normal in every respect. The copper analyses, however, revealed significant increases in the blood (39%) and spleen (19%) and very large increases in the liver (14 ×). In the rats receiving larger doses of copper the growth and food intake were markedly depressed in all cases. The rats receiving 2000 ppm. in the diet made virtually no growth while the rats on 4000 ppm. lost weight rapidly and died. In the latter case the deaths are partly due to voluntary starvation since the actual copper intake was less than in the preceding groups. The food intake was insufficient to maintain the body weight as shown by Franke and Potter ('34). The results in the group on 4000 ppm. show the necessity of considering the effect of taste in determining the results obtained on a toxic diet. It is not possible to study copper

toxicity adequately by feeding the daily dosage in a small portion of the diet.

The application of the method of Fischer and Leopoldi for the analysis of copper in biological material has made possible the analysis of individual samples of blood, liver and spleen. The results obtained with blood and spleen are in marked contrast to those obtained with liver. Whereas the higher levels of copper increased the copper content of blood and spleen a maximum of five times, the copper content of the livers of the rats receiving 2000 ppm. was raised about 300 times. In the latter case the livers were noticeably jaundiced. The results obtained with 500 ppm. check quite closely with those of Lindow, Peterson and Steenbock ('29), and of Flinn and Inouye ('29). Our results are at variance with certain data published by Cunningham ('31). This author has reported an increase of only 1.5 to 3 times in the copper content of livers of rats fed 7.5 mg. copper per day, whereas the present work and that of Lindow, Peterson and Steenbock ('29) showed an increase of 14 to 20 times in the case of livers of rats fed 5.0 mg. copper per day. Our results illustrate the marked ability of the liver to take up extremely large amounts of ingested copper. Apparently the liver has a much greater ability than blood to retain copper.

These results indicate that copper does not begin to exhibit toxic effects in the case of the rat until at least 150 times the therapeutic dosage has been reached. Apparently the copper content of the liver can be increased as much as fourteen times over a short period of time without obvious damage. It is unlikely that this amount of copper will be ingested either in practical rations or in therapeutic doses. The liver is apparently the organ which plays a predominant role in determining the amount of copper which is to remain in the body.

It is difficult to conclude from this work the location of the toxic action of copper. However, it is interesting to note that the liver shows a large increase with each increase in intake while the blood shows the most pronounced increase between 500 and 1000 ppm., and this is the range in which copper becomes definitely toxic.

SUMMARY

1. White rats were fed ad libitum diets which contained 0, 500, 1000, 2000 and 4000 ppm. of added copper in the form of $CuSO_4$.

2. The rats ingested voluntarily amounts of copper ranging from 5.05 to 11.8 mg. copper per day, although at 4000 ppm., food intake was so restricted as to result in partial starvation and rapid death.

3. Slight toxicity was observed on 500 ppm. with increasing toxicity on higher levels, as indicated by growth records.

4. The animals were killed after 4 weeks and the blood, spleens and livers were analyzed for copper. Whereas the copper content of the blood and spleens was increased a maximum of 2 to 5 times, the liver increased to a maximum of 300 times normal.

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STUDIES ON THE VITAMIN B_1 REQUIREMENTS OF GROWING CHICKS [1, 2]

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THREE FIGURES

(Received for publication October 18, 1937)

Recent reviews (Hogan and Boucher, '33; Jukes, '37) on the nutritional essentials of the chick reveal a paucity of data on the quantitative requirement of this species for the anti-neuritic vitamin. Kline, Keenan, Elvehjem and Hart ('32), Elvehjem et al. ('32), Keenan et al. ('34), and Elvehjem ('35, '36) have reported that the chick may successfully be used for studies on this vitamin. The autoclaved natural grain ration (ration 242A) developed by these investigators (Keenan et al., '34) has afforded a rapid and simple means of assay for vitamin B_1 with the day-old chick. Since fairly normal rates of growth are obtained when the above ration is supplemented with adequate amounts of vitamin B_1, it was important to establish the requirement of this species using both the international standard and crystalline vitamin B_1 hydrochloride. There is some quesion about the manner of expressing the requirement since it is related both to body weight and carbohydrate metabolism. An expression per unit weight of feed is most valuable from a practical point of view. At some stages of growth the vitamin B_1 intake on this

[1] Published with the perimission of the director of Wisconsin Agricultural Experiment Station.

[2] Presented at the ninety-fourth meeting of the American Chemical society at Rochester, September 1937.

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basis will be higher than the requirement but the method will measure the requirement at the most critical period. This procedure may readily be adapted to a determination of the antineuritic potency of vitamin B_1-containing substances.

EXPERIMENTAL

For these experiments, day-old White Leghorn chicks with an initial weight of about 35 gm. were obtained from the University Poultry Department. The chicks were placed in cages equipped with wire screen bottoms (two meshes to the inch) and suitable warmers. Each group usually contained four chicks. Water was supplied in porcelain cups daily. Weights were recorded weekly.

The basal vitamin B_1 low ration (ration 242A), modified by Keenan, Kline, Elvehjem and Hart ('34), was used throughout. Ration 242A has the following composition:

Autoclaved portion:	
Ground yellow corn	57
Pure flour middlings	25
Crude domestic acid precipitated casein	12
Untreated portion:	
Vacuum desiccated whole liver substance [4]	2
Iodized salt (0.02% potassium iodide)	1
$CaCO_3$ (precipitated)	1
$Ca_3(PO_4)_2$ (precipitated)	1
Cod liver oil	1

The preparations were tested for their antineuritic potency by substitution into the ration at various levels. In each case, the smallest level of material necessary to protect all the chicks in the group over a period of 5 weeks was determined. Any material fed on the percentage basis which protected the chicks for 5 weeks was found to continue to do so indefinitely. The rations were made up fresh weekly. In addition to the basal ration the chicks received 2 drops of halibut liver oil twice weekly to insure an adequate supply of vitamin A.

[4] Wilson Laboratories, Chicago, Illinois.

While the rate of growth of the chicks during the first 2 weeks on experiment somewhat modified the minimum protective level, the vitamin B_1 requirement of the chick when expressed as per cent of the ration was remarkably constant. The results shown in figure 1 illustrate this fact. In five separate assays it was found that all of the chicks in groups 471, 490, 511, 540, and 553 were protected from polyneuritis

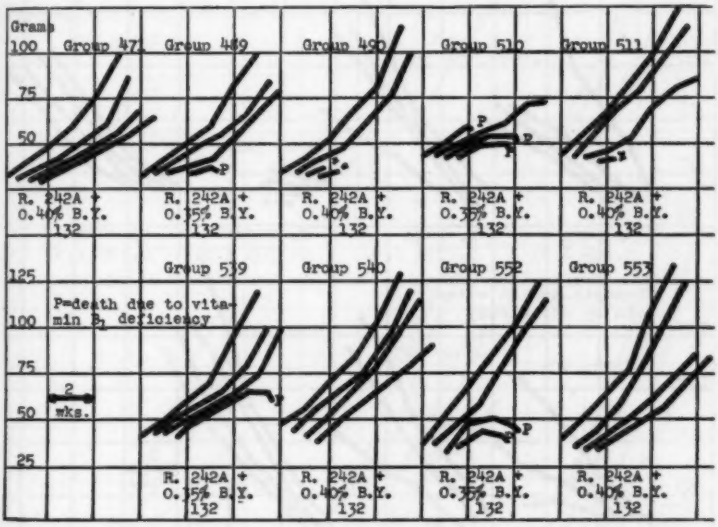

Fig. 1 Individual growth records of chicks fed ration 242A supplemented with 0.35 or 0.40% brewers' yeast 132 during a 5-week experimental period.

when the basal ration was supplemented with 0.40% brewers' yeast 132.[5] When the level of yeast was reduced to 0.35%, one or more chicks in groups 489, 510, 539, and 552 died after exhibiting the typical symptoms of polyneuritis.

It has been found that chicks are able to elute the vitamin from the international standard acid clay adsorbate. The results obtained with different levels of the international

[5] We are indebted to Dr. Harold Levine of the Premier-Pabst Corporation, Milwaukee, Wisconsin, for several generous samples of yeast.

standard are shown in figure 2. When the chicks were fed ration 242A supplemented with 0.175% international standard, all chicks in group 592 died, two showing polyneuritis, and two of the four chicks in group 604 died after exhibiting the

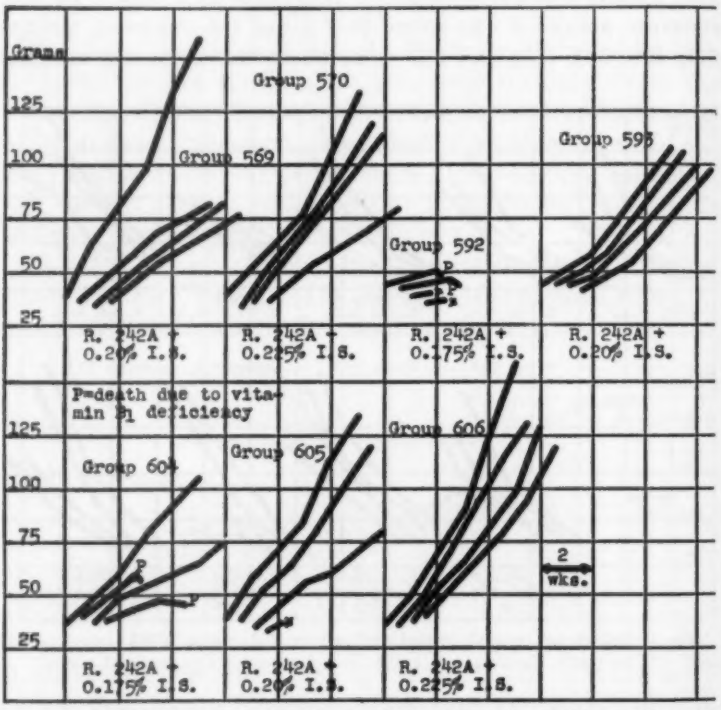

Fig. 2 Individual growth records of chicks fed ration 242A supplemented with 0.175, 0.200 or 0.225% international standard during a 5-week experimental period.

typical symptoms of polyneuritis. All of the chicks in groups 569, 593, and 605 were protected from polyneuritis when fed ration 242A supplemented with 0.20% international standard. An improved rate of growth resulted when the chicks were fed ration 242A supplemented with 0.225% international standard (groups 570 and 606). When the criterion for the

determination of the minimum protective level is taken to be protection from polyneuritis for all the chicks in the group, it is evident that the vitamin B_1 requirement of the chick is constant.

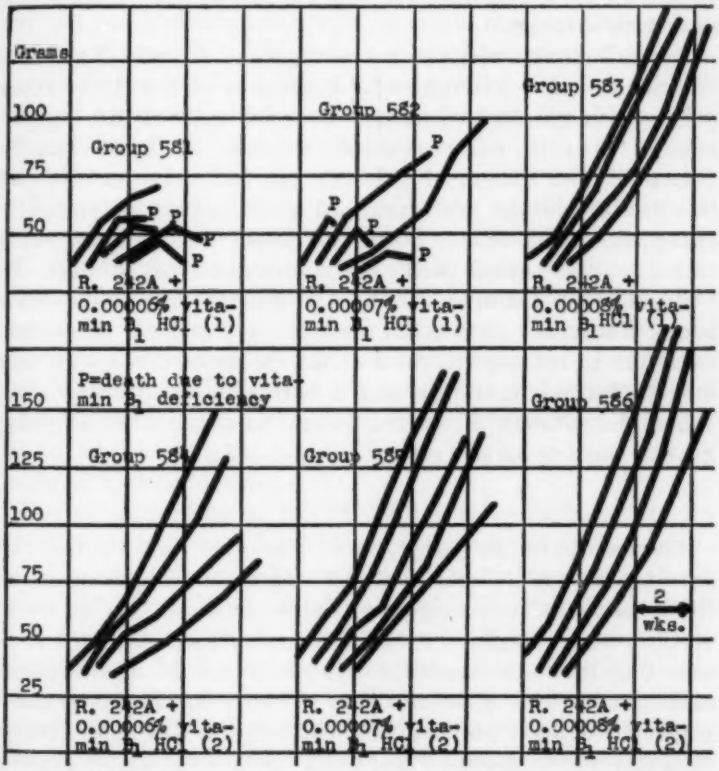

Fig. 3 Individual growth records of chicks fed 60, 70 or 80 micrograms crystalline vitamin B_1 hydrochloride (samples 1 and 2) per 100 gm. ration 242A for a 5-week experimental period.

The requirement was also established in terms of the crystalline vitamin. Two accurately weighed samples [6] of crystalline vitamin B_1 hydrochloride (synthetic) were dis-

[6] The vitamin B_1 samples were kindly weighed out by Mr. H. A. Campbell on a micro-analytical balance.

solved in 20% alcohol to give a final concentration of 100 micrograms per cubic centimeter. For feeding purposes, suitable aliquots were dried in vacuo on about 50 gm. portions of the autoclaved component of the ration. This was ground by hand before incorporation into the ration, care being taken to eliminate losses.

Typical results with the two samples of vitamin B_1 hydrochloride are shown in figure 3. Protection of the chicks from polyneuritis resulted when they were fed ration 242A supplemented with 80 micrograms of vitamin B_1 hydrochloride (sample 1) per 100 gm. of ration (group 583). Lower levels of this product did not protect all of the chicks from polyneuritis (groups 581 and 582). When the chicks were fed the basal ration supplemented with 60 micrograms of vitamin B_1 hydrochloride (sample 2) per 100 gm. of ration, complete protection from polyneuritis resulted (group 584). The wide variation in the growth rates of the chicks in this group was due to the fact that this was a borderline protective level. When the vitamin B_1 in the ration was increased slightly, growth was improved (groups 585 and 586).

DISCUSSION

The results in this paper, as well as other data obtained in our laboratory, indicate that the vitamin B_1 requirement of chicks based on unit weight of feed is constant. On the basis of the results obtained with the international standard it is seen that the requirement is between 20 and 25 international units per 100 gm. of ration 242A. Twenty international units protected against polyneuritis with several different groups of chicks on the specific basal used. Somewhat better growth resulted with 25 international units per 100 gm. of ration 242A. It is perhaps safer to assume that the normal requirement is about 25 international units since ration 242A is known to contain slight amounts of vitamin B_1. A slightly higher level may be necessary on a basal ration completely devoid of this vitamin.

A significant difference in the antineuritic potency was observed between the two samples of crystalline vitamin B_1 hydrochloride that were tested. Sample 1 was more active since 60 micrograms per 100 gm. of ration 242A protected the chicks from polyneuritis. Eighty micrograms of sample 2 per 100 gm. of ration 242A were required to protect the chicks from polyneuritis. The reason for the variation in antineuritic potency is not clear at the present time. Nevertheless, we feel justified in assuming that sample 1 yields a truer evaluation of the antineuritic potency of the crystalline vitamin.

The above data permit an evaluation of the antineuritic potency of brewers' yeast 132. Since 0.40% of brewers' yeast 132 was required to protect the chicks from polyneuritis, it follows that this yeast sample contained 50 international units per gram. Similarly, a comparison between the international standard and vitmain B_1 hydrochloride (sample 1) indicates that vitamin B_1 hydrochloride contained 330 international units per milligram. The ratio of the international unit to crystalline vitamin B_1 hydrochloride has been reviewed by Sampson and Keresztesy ('37).

The establishment of the values for the minimum vitamin B_1 requirements of chicks will greatly simplify the calculations of the vitamin B_1 content of food materials where chicks have been used for the assays. The results obtained with the chicks indicate that this method possesses certain advantages over the rat assay method. Less difficulty is experienced in the preparation of the basal ration for chicks than in the purification of material for synthetic rat rations. The data obtained with several different batches of chicks show that no difficulty is encountered with respect to storage of vitamin B_1 in the day-old chick. In addition it appears that the chick is more sensitive to small additions of the vitamin to the basal ration than the rat.

SUMMARY

1. The vitamin B_1 requirement of the chick as measured by the prophylactic technic is remarkably constant.

2. When ration 242A is supplemented with 20 to 25 international units of vitamin B_1 supplied by the international standard acid clay adsorbate the chicks are protected from polyneuritis.

3. Assays on samples of crystalline synthetic vitamin B_1 indicate that chicks are protected from polyneuritis when they are fed 60 micrograms of vitamin B_1 hydrochloride (sample 1) per 100 gm. of ration 242A. A second sample (sample 2) possessed lower antineuritic potency since 80 micrograms of crystalline vitamin B_1 hydrochloride per 100 gm. of ration 242A were required to protect the chicks from polyneuritis.

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